

Department of Biochemistry and Nutrition, Polytechnic Institute, and
Danish Fat Research Institute, Copenhagen, Denmark.

Influence of Dietary Hydrogenated Peanut Oil and Cholesterol on Cholesterol and Polyenoic Fatty Acids in Tissues of Chicks.

By

HENRIK DAM, AAGE JART, GUNHILD KRISTENSEN, GUNHILD
KOFOED NIELSEN and EBBE SØNDERGAARD.

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In previous studies we have examined the content of cholesterol and polyenoic fatty acids in various tissues of chicks fed diets containing varied levels of peanut oil and cholesterol (DAM et al. 1956, DAM and KOFOED NIELSEN 1956). These investigations have now been supplemented with a study of the influence of *hydrogenated* peanut oil.

Experimental.

Sixty-day-old chicks were reared on a "normal" commercial diet* (DAM and SØNDERGAARD 1953, Table II) for two weeks. Thereafter they were distributed into 6 groups (10 chicks in each) and given artificial diets containing 0, 10, and 20 % of hydrogenated peanut oil with and without addition of 1 % cholesterol. These diets consisted of the fat-free diet no. 3 (DAM, PRANGE and SØNDERGAARD 1955, Table I) into which the hydrogenated peanut oil and cholesterol were incorporated instead of the corresponding amount (by weight) of sucrose. Vitamins A and D were given in filtrol-treated peanut oil, 1 drop (24 mg of the oil solution) twice a week, furnishing an average of 220 I.U. vitamin A and 20 I.U. vitamin D₃ and 7 mg of peanut oil per chick per day.

* Containing 3.7 % fat. Polyenoic fatty acids determined as percent of total fat by alkali isomerization show 26.0 % dienoic, 0 % trienoic, 1.1 % tetraenoic, 0.4 % pentaenoic, 1.7 % hexaenoic acids.

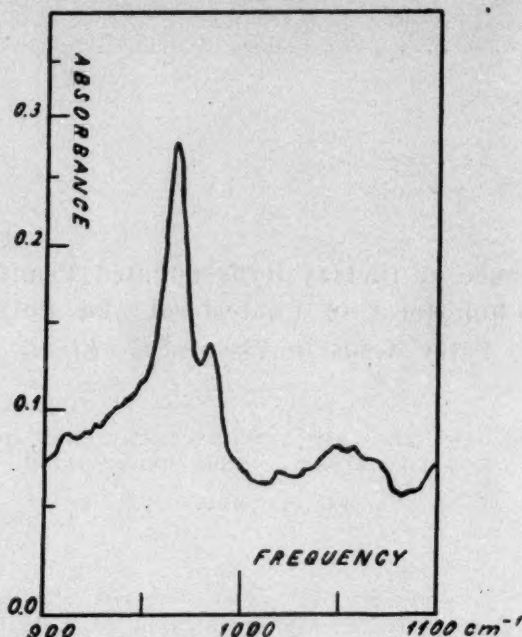


Fig. 1. Infrared spectrum of liver fatty acids from group 1708.

The chicks were sacrificed by decapitation after having received the artificial diets for 4 weeks. Cholesterol and polyenoic acids were determined as described earlier (DAM et al. 1956).

The hydrogenated peanut oil, which melted between 40 and 42° C, was also analyzed. Its content of conjugable polyenoic fatty acids was determined by the same method as used for the tissue fats (alkali-isomerization with 21 % KOH in glycol for 15 min. at 180° C (HAMMOND and LUNDBERG 1953). In order to ascertain that trans-dienoic acids were not present in an amount that would influence the calculation of the results, isomerization was also carried out with 6.5 % KOH in glycol at 180° C for 25, 60, and 285 min. (JACKSON et al. 1952). The absorption in the region about 232 $m\mu$ obtained by these treatments was not higher than that obtained by 21 % KOH at 180° C for 15 min.

The content of conjugable polyenoic acids in the hydrogenated peanut oil was therefore calculated to be 0.1 % dienoic. The content of preformed conjugated fatty acids was 0.1 % dienoic. Conjugable and preformed conjugated more unsaturated acids were not present.

The iodine number (Wijs) was 51.0, corresponding to 67.5 % monoenoic acid. The content of saturated acids determined as the difference: total fatty acids minus (monoenoic + polyenoic acids) was 32.3 %.

The content of acids with trans-double bonds, calculated as elaidic, was 46 %.

Trans-fatty acids were determined in the fatty acid fractions from the hydrogenated peanut oil and from the fat of liver and adipose tissue by infrared spectrophotometry using a double beam spectrophotometer (Perkin Elmer, Model 21). The solvent was carbon disulfide and the effect of the carboxyl group was compensated for by a solution of oleic acid. The results were calculated as isolated trans- (elaidic) acid using the molar extinction coefficient 155.9 at 10.35μ (965 cm^{-1}) (SHREEVE et al. 1950). In the case of liver fatty acids the infrared spectrum also showed a small peak at 10.20 to 10.15μ (980 — 985 cm^{-1}), cf. the example shown in Fig. 1. This smaller peak was interpreted as trans-double bonds in a conjugated system.

Results and Discussion.

Cholesterol content of tissues, Table I.

The feeding of 10 or 20 % of hydrogenated peanut oil without cholesterol did not result in a decrease of liver cholesterol, such as it had been found previously when unhydrogenated peanut oil was fed. When 10 or 20 % of hydrogenated peanut oil was fed with 1 % of cholesterol, the cholesterol content of all the organs examined, except brain, increased, but there was no significant difference between the values obtained with 10 and 20 %, respectively. In the previous experiments with unhydrogenated peanut oil and 1 % cholesterol, 20 % of oil resulted in a much higher deposition of cholesterol in the liver than did 10 %; there was also an increase in adipose tissue cholesterol when the amount of unhydrogenated peanut oil (together with 1 % cholesterol) was increased from 10 to 20 %.

The increase of cholesterol in liver, heart, aorta and fat tissue caused by feeding hydrogenated peanut oil together with cholesterol is apparently due to increased absorption of cholesterol when fat is given. In this respect 10 % hydrogenated peanut oil had approximately the same effect as 10 % unhydrogenated peanut oil.

The hypothesis according to which cholesterol is metabolized easier when polyenoic acids are fed finds support in our foregoing experiments (DAM et al. 1956) only as far as certain tissues, especially liver, is concerned and only when 0 or 0.1 % cholesterol was fed.

Table I.
Content of cholesterol, expressed as mg per 100 g tissue (wet weight, except in the case of aorta).¹

Group no.	1703	1704	1705	1706	1707	1708
Additions to diet:						
% hydrogenated peanut oil	0	0	10	10	20	20
% cholesterol	0	1	0	1	0	1
Liver	404 ^a ± 22	684 ^a ± 54	432 ^a ± 21	2,190 ^a ± 900	428 ^a ± 10	2,048 ^a ± 229
Aorta (dry weight)	706 ^a ± 40	949 ^a ± 75	662 ^a ± 29	1,544 ^a ± 165	712 ^a ± 22	1,918 ^a ± 256
Heart	163 ^a	207 ^a	175 ^a	437 ^a	189 ^a	328 ^a
Brain	1,210 ^a	1,207 ^a	1,254 ^a	1,233 ^a	1,219 ^a	1,248 ^a
Fat tissue	103 ^a	103 ^a	104 ^a	155 ^a	86 ^a	126 ^a
Weight of animals, grams ²						
initial	110 ± 5	112 ± 5	111 ± 5	112 ± 6	111 ± 6	112 ± 5
final	270 ± 23	270 ± 18	267 ± 16	276 ± 13	263 ± 17	241 ± 18

¹ All figures are averages. The superscripts indicate the number of organs examined. In the case of liver and aorta, the cholesterol determinations were carried out for each animal in the group. The standard error is indicated by \pm . In the case of heart, brain, and fat tissue, four or five organs were pooled for each determination.

² Each group consisted of 10 animals.

Table II.

Polyenoic fatty acids, conjugable by heating with 21 % KOH in glycol for 15 min. in per cent of total fatty acids in tissues of chicks fed experimental diets for 4 weeks.

Group no.		1703	1704	1705	1706	1707	1708
Additions to diet:	% hydrogenated peanut oil	0	0	10	10	20	20
	% cholesterol	0	1	0	1	0	1
Liver ¹	% dienoic	1.3	2.9	3.5	1.9	2.9	1.7
	% trienoic	2.8	3.8	1.9	1.1	1.3	0.7
	% tetraenoic	2.2	3.4	2.9	1.5	2.0	1.2
	% pentaenoic	0.2	0.4	0.5	0.2	0.5	0.3
	% hexaenoic	0.4	0.8	1.2	0.3	0.7	0.3
	% total	6.9	11.3	10.0	5.0	7.4	4.1
Heart ²	% dienoic	5.8	6.7	6.3	5.9	6.2	7.5
	% trienoic	4.6	4.2	1.6	1.3	1.3	1.1
	% tetraenoic	4.8	4.5	4.0	3.6	3.8	3.6
	% pentaenoic	0.3	0.3	0.3	0.3	0.3	0.3
	% hexaenoic	0.2	0.2	0.2	0.2	0.1	0.2
	% total	15.7	15.9	12.4	11.3	11.7	12.7
Brain ²	% dienoic	-3.4	-3.0	-1.8	-1.8	-1.4	-1.8
	% trienoic	1.1	2.1	3.2	3.6	3.6	2.7
	% tetraenoic	3.9	5.1	7.6	8.7	8.2	8.0
	% pentaenoic	2.0	2.3	2.6	2.9	2.5	2.7
	% hexaenoic	4.1	5.0	6.8	8.7	7.9	7.3
	% total*	7.7	11.5	18.4	22.9	20.8	18.9
Fat tissue ³	% dienoic	1.9	2.6	1.4	1.7	1.4	1.4
	% trienoic	0.3	0.4	0.1	0.2	0.1	0.0
	% tetraenoic	0.0	0.0	0.0	0.1	0.0	0.0
	% pentaenoic	0.0	0.0	0.0	0.0	0.0	0.0
	% hexaenoic	0.1	0.0	0.0	0.1	0.0	0.0
	% total	2.3	3.0	1.5	2.1	1.5	1.4

¹ Two livers were pooled for each analysis. The figures are averages of five determinations.

² Four or five organs were pooled for each analysis. The figures are averages of two determinations.

* The negative values are subtracted from the total fatty acids.

Polyenoic fatty acids (expressed as percentages of total fatty acids), Table II.

The most important finding seems to be that in liver and heart the percentage of trienoic acid was decreased by the addition of 10 and 20 % hydrogenated peanut oil to the diet, just as it was found in the previous experiments with unhydrogenated peanut oil.

Table III.

Per cent trans-fatty acids of total fatty acids, expressed as per cent elaidic acid.

Group no.	Diet characteristics		Liver		Fat tissue	
			Per cent trans-fatty acids			
			iso-lated	con-jugated	iso-lated	con-jugated
1703	fat-free	+ 0% cholesterol	0	0	0	0
1704	fat-free	+ 1% cholesterol	0	0	0	0
1705	10% hydrogenated peanut oil	+ 0% cholesterol	18	4	26	0
1706	10% hydrogenated peanut oil	+ 1% cholesterol	18	3	24	0
1707	20% hydrogenated peanut oil	+ 0% cholesterol	23	5	31	0
1708	20% hydrogenated peanut oil	+ 1% cholesterol	19	4	32	0

A similar effect of hydrogenated peanut oil on trienoic acid was not found for brain, contrary to what was the case when unhydrogenated peanut oil was given.

The figures for the individual polyenoic acids for brain are much more uncertain than those found for the other organs, and negative values were found for brain dienoic acid. This must be due to the fact that the set of formulae used for the calculations is not strictly applicable to brain fatty acids. According to KLENK and LINDLAR (1955 a, b), the chain lengths of the polyenoic acids in the glycerophosphatides of brain are not the same as those on which the calculation is based (pentaenoic assumed to be C_{22} , HAMMOND and LUNDBERG 1953).

In the fat tissue, where the only polyenoic acid was dienoic which occurred in a small amount, no differences were observed.

Trans-fatty acids in liver and fat tissue, Table III.

Feeding of hydrogenated peanut oil has caused deposition of fatty acids with isolated trans-double bonds in liver and fat tissue, and in addition thereto, a much lesser deposition of conjugated trans-acids in the liver.

Table IV.

Iodine values and content of monoenoic acid and saturated acids in per cent of total fatty acids in liver, calculated as if trans-polyenoic acids were not present.

Group no.	1703	1704	1705	1706	1707	1708
Additions to diet:						
% hydrogenated peanut oil	0	0	10	10	20	20
% cholesterol	0	1	0	1	0	1
Iodine value	62.4	67.7	85.6	79.3	85.7	76.6
% monoenoic acid	49.1	48.4	65.3	69.7	71.0	69.7
% saturated acids	43.4	39.2	21.5	22.4	18.2	23.4

Table V.

Daily consumption of diet. Grams of diet consumed per chick per day during each of the 4 weeks of experimental feeding.

Group no.	Additions to diet:		1. week	2. week	3. week	4. week
	hydrogenated peanut oil %	cholesterol %	g	g	g	g
1703	0	0	11.0	15.8	21.4	21.2
1704	0	1	11.7	15.4	19.4	21.5
1705	10	0	11.1	13.2	17.4	18.6
1706	10	1	9.8	14.9	16.9	18.0
1707	20	0	13.2	15.4	16.6	18.9
1708	20	1	11.4	15.6	15.5	17.2

Iodine values of liver fat, Table IV.

Ten and twenty per cent of hydrogenated peanut oil have caused an increase of the iodine value of the total liver fatty acids and to approximately the same extent. Monoenoic acids were increased and saturated acids decreased.

If trans-polyenoic acids occur, the values for monoenoic acids are lower and those for saturated acids higher than indicated in the table.

The finding that 10 and 20 % hydrogenated peanut oil gave the same amount of monoenoic acids in the liver might be related to the finding of the same amount of cholesterol in these two cases.

Daily consumption of diet, Table V.

From the data in Table V it can be seen that the values obtained for tissue cholesterol and tissue fatty acids in the various groups cannot be due to variations in the amount of food consumed.

Summary.

The influence of dietary hydrogenated peanut oil and cholesterol on cholesterol and polyenoic acids in various tissues of chicks was examined and compared with the results of earlier studies with unhydrogenated peanut oil.

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Institute of Medical Biochemistry and Physiology, Department of
Medical Biochemistry, University of Oslo, Norway.

Acid Soluble Mononucleotides in Rat Diaphragm During Incubation.

By

AA. RYE ALERTSEN, O. WALAAS and E. WALAAS.

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Indications have been obtained that stimulation of glucose uptake in rat diaphragm by insulin depends upon adequate energy production by respiration and phosphorylations. Thus, the insulin effect on rat diaphragm is reduced or abolished under anaerobic conditions (WALAAS and WALAAS 1952, HAFT and MIRSKY 1952, DEMIS and ROTHSTEIN 1954, OTTAWAY 1955), by addition of 2,4-dinitrophenol (JERVELL, WALAAS and WALAAS 1956) or NaCN (E. WALAAS 1955). Further, RESNICK and HECHTER (1957) have reported that insulin stimulation of galactose transfer in rat diaphragm is suppressed by dinitrophenol, dinitronaphthol and iodoacetate. These observations indicate that insulin stimulation of hexose transport across muscle cell surface structure (LEVINE and GOLDSTEIN 1955) depends upon processes which require generation of high energy phosphorus compounds.

Abbreviations:

AMP — Adenosine-5'-monophosphate
ADP — Adenosine diphosphate
ATP — Adenosine triphosphate
DNP — Diphosphoryridine nucleotide
IMP — Inosine-5'-monophosphate

GTP — Guanosine triphosphate
UTP — Uridine triphosphate
FAD — Flavine adenine dinucleotide
2,4-DNP — 2,4-dinitrophenol

Therefore, in the course of studies on insulin effect on glucose uptake by muscle, it is desirable to obtain information on the content of different mononucleotides during incubation. Previous investigations on the content of acid soluble phosphorus compounds of rat diaphragm have been made by the relatively unspecific method of acid hydrolysis (HAUGAARD, MARSH and STADIE 1951, SACKS and SINEX 1952, WALAAS and WALAAS 1952, JERVELL, WALAAS and WALAAS 1956).

In the present work, the method of chromatography on anion exchange resin by the gradient elution technique has been adopted for fractionation of acid soluble mononucleotides in rat diaphragm. The work includes quantitative estimation of the different mononucleotides in rat diaphragm. Further, the interconversion and degradation of mononucleotides under several different experimental conditions have been studied.¹

Material and methods.

Incubation procedure and preparation of PCA extract of rat diaphragm. Grown rats weighing 200 to 300 g were used. Hemidiaphragms from 20 rats, the total weight being 6 to 7 g, were removed and pooled. The diaphragms were gently blotted on filter paper, weighed and kept in 50 ml ice cold Krebs Ringer phosphate medium for 15 min. Next the diaphragms were transferred to a 100 ml Erlenmeyer flask containing 20 ml Krebs Ringer phosphate medium pH 7.4 with 140 mg glucose per 100 ml, and incubated at 37° C in a Warburg bath with shaking. In aerobic experiments the flasks were equilibrated with 100 per cent oxygen. In anaerobic experiments argon of purity 99.56 per cent was used. Prior to incubation the small amount of oxygen was removed by passing the gas through an alkaline solution of pyrogallol and then over heated copper. When incubation was finished the Erlenmeyer flask was chilled in an ice bath, the diaphragms were rapidly removed, dried on filter paper and transferred to a mortar containing 2 volumes of ice cold 0.6 N PCA. The tissue was ground with equal amounts of sand and extracted for 1 hour at + 2° C. The precipitate was spun down in an "International" Refrigerated centrifuge for 15 min at 10,000 $\times g$. The supernatant was decanted and the residue reextracted by the same procedure. The combined supernatants were neutralized to pH 6 to 7 with 5 N KOH and finally with 1 N KOH. After precipitation in the cold over night potassium perchlorate was removed by centrifugation. The protein free extract thus obtained was used for ion-exchange chromatography.

¹ A preliminary report of this investigation has been published (ALERTSEN, WALAAS and WALAAS 1957).

Ion-exchange chromatography. Dowex-1, X-8, 200–400 mesh, manufactured by Dow Chemical Company, Midland, Michigan, was prepared in the formate form. Elution was performed by the technique of HURLBERT et al. (1954). Columns 10 cm \times 1 cm were prepared and chromatography was performed at $+2^{\circ}\text{C}$ in a cold room by gradient elution. The rate of elution was 0.28 ml per min and fractions of approximately 5 ml were collected in an automatic time operated fraction collector manufactured by LKB, Stockholm, Sweden. Every fraction was read at 260 $m\mu$ and 275 $m\mu$ in a Beckman spectrophotometer model DU. For further identification appropriate fractions were pooled and concentrated either by lyophilization or by treatment with norite by the method of BERGQUIST (1956).

Paper ionophoresis. Paper ionophoresis was performed in an apparatus described by FOSTER (1952) on Whatman no. 1 filter paper in 0.02 M citrate buffer at pH 3.5 as described by DAVIDSON and SMELLIE (1952). The nucleotides were located in ultraviolet light photographically according to the method of MARKHAM and SMITH (1949). For analytical investigation the spots were cut out and eluted with 0.1 N HCl for 30 min. Blank areas were cut out, eluted and used as controls. The eluates were used for ultraviolet spectrophotometry and other analytical procedures. If necessary the eluates were concentrated by lyophilization.

Analytical procedures. For identification of the different compounds the following procedure was used routinely:

Location by ion-exchange chromatography.

Location by paper electrophoresis.

Ultraviolet absorption spectrum and the ratio of absorbancy E_{275}/E_{260} .

Determinations of phosphorus compounds.

Determination of ribose.

In some instances more special analytical methods, as mentioned below, were used. As reference standards a series of commercially obtained mononucleotides were chromatographed and subjected to the procedure mentioned. These standards included the mono-, di- and triphosphates of the common purin- and pyrimidin-nucleosides. These compounds were derived from "Sigma" Chemical Company, except ADP and GTP which were obtained from Nutritional Biochemical Corporation.

Optical densities were measured on Beckman spectrophotometer model DU and quantitative estimations were based upon the following molar absorbancy (Pabst Bulletin 1956).

Adenosine phosphates

14.7×10^3 at 257 $m\mu$, pH 2 (MORELL and BOCK 1954).

Guanosine phosphates

12.4×10^3 at 256 $m\mu$, pH 11 (VOLKIN and COHN 1957).

Inosine phosphates

7.4×10^3 at 260 $m\mu$, pH 2 and pH 7.

12.4×10^3 at 250 $m\mu$, pH 2 and pH 7 (VOLKIN and COHN 1957).

Uridine phosphates

10.0×10^3 at 262 $m\mu$, pH 2 PLOESER and LORING 1949).

Cytidine phosphates

 13.0×10^3 at 280 m μ , pH 2 (VOLKIN and COHN 1957).DPN 18.0×10^3 at 259 m μ , pH 7 (KORNBERG and HORECKER 1953).

Phosphorus was determined by the method of FISKE and SUBBAROW (1925) with aid of a Hilger colorimeter, filter no. 70. If small amounts of material was available phosphorus determination was made on Beckman spectrophotometer at 660 m μ using the micro equipment designated by LOWRY and BESSEY (1946). Determination of inorganic P, acid labile P (10 min hydrolysis in N sulfuric acid) and total P was done.

Pentose was measured by the method of HURLBERT et al. (1954). In addition, pentoses and purine-ribosides were determined by the method of DISCHE and BORENFREUND (1957).

Identification of DPN was made by the method of cyanide complex formation (COLOWICK, KAPLAN and CIOTTI 1951), as well as by enzymatic assay with alcohol dehydrogenase (KAPLAN 1955).

Estimation of 5'-substitution of ribo-nucleotides was carried out by oxidation with periodate by the method of DIXON and LIPKIN (1954).

FAD was determined by fluorescence measurement according to the method of BESSEY, LOWRY and LOVE (1949) on a Farrand spectrofluorometer.

For investigations of labile bound carbohydrates the nucleotides were subjected to hydrolysis in 0.01 N HCl, or N HCl followed by ionophoresis on paper according to the method of CONSDEN and STANIER (1952) and application of the spraying technique described by PARTIDGE (1949).

By the present procedure of ion-exchange chromatography a recovery of 98–100 per cent was obtained with adenine nucleotides. With GTP and UTP recovery was of the order 80–85 per cent. Apparently some destruction occurred during lyophilization of these components.

By determinations of nucleotides in the rat diaphragm in corresponding experiments, individual variations of concentration of the components were of the order ± 10 per cent.

Radioactive P^{32} was obtained as carrier free sodium phosphate from the Joint Establishment For Nuclear Energy Research, Kjeller, Norway.

Measurement of radioactivity was done by the counting device FH 49 from Frieske & Hoepfner, Erlangen-Bruck. The measurements were done on samples applied on paper strips, by use of an automatic scanning device coupled to a continuous recorder.

Results.

Initial content of mononucleotides in rat diaphragm. By the chromatographic procedure 8 different peaks were separated from PCA extracts of rat diaphragm (Fig. 1). In comparison it is seen that 6 peaks are obtained from extracts of rat leg muscle. Tenta-

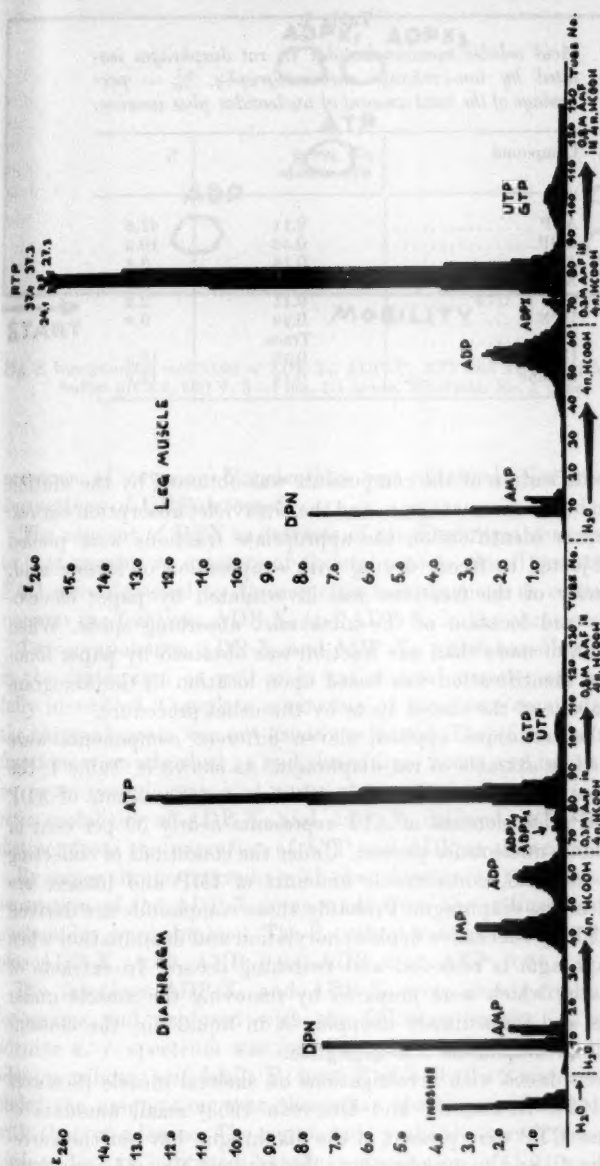


Fig. 1. Ion-exchange chromatography of PCA extracts from rat diaphragm and rat skeletal leg muscle, Dowex-1 X-8 (200-400 mesh), column 1 cm \times 10 cm in formic acid system. Mixer: 250 ml. Reservoir: 4 N at the beginning, changed as indicated. Fractions: Every 18 minutes. Rate 0.25 ml per minute. The E_{240} was determined directly on the eluates.

Table I.

Acid soluble mononucleotides in rat diaphragm isolated by ion-exchange chromatography. % = percentage of the total amount of nucleotides plus inosine.

Compound	μM per g wet muscle	%
ATP	2.11	42.6
ADP	0.52	10.5
AMP	0.16	3.2
ADP-X ₁ + ADP-X ₂	0.06	1.2
GTP + UTP	0.11	2.2
DPN	0.49	9.9
FAD	Trace	
IMP	0.77	15.5
Inosine	0.74	14.9

tive identification of the components was obtained by the elution position in the chromatogram and the ultraviolet absorption curves. For further identification the appropriate fractions were pooled and subjected to freeze drying with evaporation of formic acid. The purity of the fractions was investigated by paper electrophoresis and location of the ultraviolet absorbing spots. When indication of more than one fraction was obtained by paper ionophoresis, identification was based upon location in the ionogram and analysis of the eluted spots by the usual procedure.

By the technique applied, eleven different components were identified in extracts of rat diaphragm. As shown in Table I, the bulk is composed of ATP and in addition small amounts of ADP and AMP. The content of ATP represents nearly 50 per cent of acid soluble nucleotides present. Under the conditions of collecting muscle material, considerable amounts of IMP and inosine are present in the diaphragm. Probably these compounds are derived from ATP by successive dephosphorylation and deamination when the diaphragm is removed and twitching occurs. In extracts of leg muscle, which were prepared by removing the muscle under narcosis and immediately dropping it in liquid air, the content of inosinic compounds was negligible.

In accordance with investigations on skeletal muscle (SCHMITZ et al. 1954, BERGQUIST and DEUTSCH 1953) small amounts of GTP and UTP were present in the diaphragm, but not the corresponding di- and mono-phosphate derivatives. No evidence of the

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Fig. 2.

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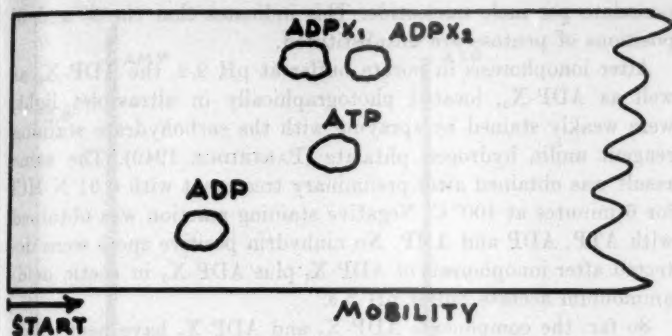


Fig. 2. Ionophoretic mobilities of ADP-X₁, ADP-X₂, ATP and ADP. 0.02 M citrate buffer pH 3.5, 600 V, 3-4 ma, 1.5 hours. Whatman No. 1 paper.

presence of cytosine 5' nucleotides was obtained. Nor were any derivatives of UDP detected.

The content of DPN in extracts of rat diaphragm corresponded to that present in extracts of skeletal muscle. Small quantities of FAD were detected by fluorometric measurement as contaminations to the fractions ADP-X₁ and ADP-X₂ in the chromatogram.

Two components, ADP-X₁ and ADP-X₂, which have been found in the diaphragm as well as in rat skeletal muscle, are not yet fully identified. Complete separation of these two compounds on the chromatogram was not usually achieved. Therefore, the mixed fractions were adsorbed on and eluted from norite and investigated by paper-ionophoresis and paper chromatography. The ionophoretic mobilities of ADP-X₁ and ADP-X₂ differed and were also distinct from the migration of ATP and ADP as shown in Fig. 2.

By paper chromatography with phenol water (4:1) as the solvent separation of the ADP-X compounds from the ordinary adenine nucleotides were obtained. The R_F values were as follows, ADP-X₁ plus ADP-X₂: 0.30, AMP: 0.46, ADP: 0.12, ATP: 0.04.

The fractions ADP-X₁ and ADP-X₂ were eluted from paper ionograms and analyzed with the following results. A typical adenine u. v. spectrum was found at pH 2 and pH 12.7. A ratio adenine:ribose:acid labile P:total P of 1:1:1:2 was observed under the assumption that the molar absorptancy was identical with that of adenine. The compounds probably were 5'phosphate nucleotides, as indicated by the consumption of 1.05 moles of

periodate per mole nucleotide. This indicates that the 2' and 3' positions of pentose are unsubstituted.

After ionophoresis in borate buffer at pH 9.2, the ADP- X_1 as well as ADP- X_2 , located photographically in ultraviolet light, were weakly stained by spraying with the carbohydrate staining reagent anilin hydrogen phthalate (PARTRIDGE 1949). The same result was obtained after preliminary treatment with 0.01 N HCl for 6 minutes at 100° C. Negative staining reaction was obtained with ATP, ADP and AMP. No ninhydrin positive spots were detected after ionophoresis of ADP- X_1 plus ADP- X_2 in acetic acid/ammonium acetate buffer pH 3.5.

So far, the components ADP- X_1 and ADP- X_2 have been characterized as complexes, stainable by a carbohydrate staining reagent, a property also observed after mild hydrolysis.

The "turnover" rate of phosphate into these compounds was studied in an experiment where 50 μ C radioactive P^{32} as carrier free inorganic phosphate was added to the incubation medium. Incubation of the diaphragms was done for 30 minutes, followed by extraction and chromatography by the usual procedure. After lyophilization, measurement of radioactivity of the different fractions was done on paper strips by an automatic scanning technique by means of a recorder. Incorporation of P^{32} into ADP- X_1 plus ADP- X_2 was very low, the radioactivity in these components being 330 counts per min per μ M compared with 14,600 counts per min per μ M in ATP. Thus any particular significance of the ADP-complexes in metabolic reactions has not been shown.

The possibility that the complexes of ADP were "artefacts" formed by conversion of other nucleotides during the procedure of extraction and chromatography was considered. By chromatography of 10 μ M commercially obtained ADP, a component amounting to 0.6 μ M was located on the chromatogram corresponding to the ADP-complexes. However, no such fraction was obtained by chromatography of pure ADP or ATP, even if these compounds were subjected to treatment with PCA and neutralization with KOH before the chromatographic procedure.

The possibility that ADP-complexes were formed from DPNH during the chromatographic procedure was investigated. 10 μ M DPNH was dissolved in PCA, neutralized with KOH and subjected to chromatography. As shown in Fig. 3 three different peaks were obtained from DPNH. In addition to a very small amount of DPN (I) a fraction amounting to 2.6 μ M with adenine

Fig.
column

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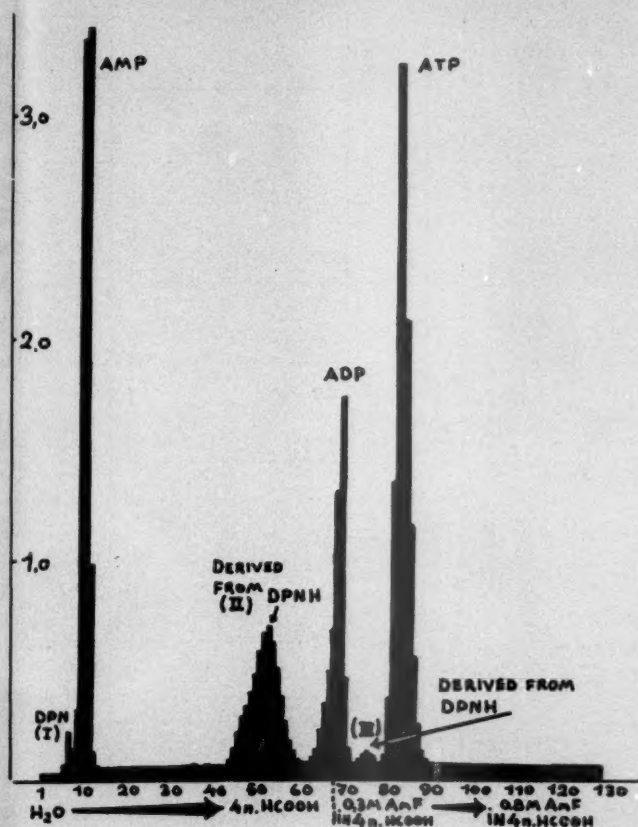


Fig. 3. Ion-exchange chromatography of AMP, ADP, ATP and DPNH on Dowex-1 column. The components were dissolved in PCA, neutralized with KOH before application to the column. Conditions as given in Fig. 1.

u. v. spectrum was found (II). This component had a ratio adenine: total P of 1 : 1 but the location on the chromatogram was different from AMP. In addition 0.46 μ M of a fraction (III) with location corresponding to ADP-X complexes was recovered. The u. v. absorption spectrum was identical with that of adenine and electrophoretically the migration corresponded with the mobility of ADP-X₁. However, the identity of this last mentioned product

Table II.
The content of acid soluble mononucleotides in rat diaphragm after incubation under aerobic conditions.

Conditions	Incubation time in minutes	μ M per g wet diaphragm							Incine	
		ATP	ADP	AMP	DPN	GTP + UTP	ADP-X ₁ + ADP-X ₂	FAD		
									Diaphragm	Medium
Initial	0	2.11	0.52	0.16	0.49	0.11	0.06	Trace	0.74	
Aerobic	30	1.49	0.31	0.09	0.23	0.09	0.08	Trace	1.09	1.25
Aerobic + 0.5 unit insulin per ml	30	1.33		0.06	0.23	< 0.05	< 0.05		0.54	
Aerobic + 2 μ g Adrenalin per ml	30	1.28	0.14	0.06	0.23	0.09	< 0.05		0.69	

Table III.

Equilibrium AMP:ADP:ATP in rat diaphragm. Incubation in aerobic and anaerobic conditions and after addition of 2,4-DNP.

Conditions	Ratio:			ATP μ M per g muscle
	AMP	ADP	ATP	
Diaphragm initial	1	3.3	13.2	2.11
Diaphragm incubated for 30 minutes aerobic	1	3.4	16.6	1.49
Diaphragm incubated for 30 minutes anaerobic	1	3.0	3.5	0.28
Diaphragm incubated with 2,4-DNP for 30 minutes	1	1.4	3.0	0.33
Leg muscle initial	1	6.6	56.6	5.96

derived from DPNH with ADP- X_1 complex has not yet been definitely established.

The level of mononucleotides in rat diaphragm after incubation. In these experiments the diaphragm and the incubation medium were analyzed separately for the presence of nucleotides by the chromatographic procedure.

Rat diaphragm. By incubation under aerobic conditions for 30 min a small reduction of the content of ATP occurred (Table II). Thus, no resynthesis of adenine nucleotides at the expense of the inosinic acid initially present took place. On the contrary, an increase of inosine occurred, approximately corresponding to the breakdown of ATP. The equilibrium of ATP:ADP:AMP after incubation was maintained almost unchanged as compared with the ratio initially present (Table III). No pronounced changes of the content of the other nucleotide fractions were detected. In Table II experiments where insulin or adrenaline were added to the medium during incubation are also included. By addition of 0.5 unit insulin per ml medium the pattern of nucleotides was not influenced after incubation for 30 min. Particularly, no significant effect on the level of ATP was observed. Similarly 2 μ g adrenaline had no effect on the level of nucleotides during incubation.

In anaerobic experiments the content of ATP rapidly declined to a very low level (Table IV). After 30 minutes of incubation a residual value of 10 per cent of the initial content of ATP was present. Obviously, the situation reflects a marked ATPase activity

Table IV.
The content of acid soluble mononucleotides in rat diaphragm by incubation under anaerobic conditions and after addition of NaCN or 2,4-DNP.

Conditions	Incubation time in min	μM per g wet muscle							IMP	Inosine	
		ATP	ADP	AMP	DPN	GTP + UTP	ADP-X ₁ + ADP-X ₂	Diaphragm		Medium	
Initial	0	2.11	0.52	0.16	0.49	0.11	0.06	0.77	0.74		
Anaerobic	30	0.28	0.24	0.08	0.17	< 0.05	< 0.05	1.08	1.93	1.96	
$1 \times 10^{-4}\text{M}$ NaCN	15	0.42	0.27	0.21	0.10	< 0.05	< 0.05	1.30	1.41	0.99	
$5 \times 10^{-4}\text{M}$ 2,4- DNP	30	0.33	0.15	0.11	0.19	< 0.05	< 0.05	1.47	1.40	1.43	

in the surviving rat diaphragm. A pronounced breakdown of ATP also occurred when 1×10^{-3} M NaCN or 5×10^{-3} M 2,4-DNP was present in the medium during incubation. ADP and AMP were not broken down to the same extent and the equilibrium of adenine nucleotides in the diaphragm was markedly changed compared with aerobic experiments (Table III). The fractions GTP plus UTP and ADP- X_1 plus ADP- X_2 were also reduced in anaerobiosis and only trace quantities could be recovered when the incubation was finished. The breakdown of ATP and other nucleoside phosphates in these experiments was reflected in a greatly increased accumulation of IMP plus inosine in the diaphragm.

Nucleotides in the incubation medium. By chromatographic analysis of the medium after incubation, no liberation of nucleotides from the diaphragm into the medium could be detected. However, the medium contained considerable amounts of inosine. Thus, about half of the inosine formed during incubation was recovered from the medium. Identification of inosine was done after passing extracts of the medium through a Dowex-1 column to remove ribose phosphates. By ultraviolet spectrophotometry and determination of purine riboside in the eluate by the method of DISCHE and BORENFREUND (1957) a ratio hypoxanthin : ribose of 1 : 1 was found indicating the presence of inosine. In addition, trace quantities of xanthin and uric acid were recovered from the medium. These compounds were absorbed on Dowex-1 column and identified after elution and treatment with norite by u. v. spectrophotometry.

A balance scheme of nucleotide conversion in incubation experiments with rat diaphragm is presented in Table V. Roughly, the breakdown of ATP and other nucleotides present in the diaphragm corresponds to the formation of IMP plus inosine. In these calculations a common mean value was used for the initial content. The initial value is subjected to considerable variations and may explain the discrepancy found in some of the experiments.

Discussion.

The pattern of acid soluble nucleotides in rat diaphragm is characterized by a predominance of adenine nucleotides (nearly 60 per cent), small amounts of GTP and UTP (about 1 per cent of

Table V.

Balance scheme of mononucleotides in rat diaphragm after incubation under aerobic and anaerobic conditions.

Incubation	μM per g wet muscle			
	Decrease of adenin-nucleotides ¹	Formation of		
		(I) IMP	(II) Inosine	(I + II) Total
30 min Aerobic	1.1	0	1.6	1.6
15 min Anaerobic	2.2	0.4	1.5	1.9
30 min Anaerobic	2.6	0.3	3.2	3.5
15 min Addition of 1×10^{-3} M NaCN	2.3	0.5	1.7	2.3
30 min Addition of 5×10^{-3} M 2,4-DNP	2.6	0.7	2.1	2.8

¹ Including DPN and $\text{ADP-X}_1 + \text{ADP-X}_2$.

each) and a somewhat higher content of DPN (10 per cent). A similar distribution has been shown in rat skeletal muscle (SCHMITZ et al. 1954, and the present work), and in rabbit skeletal muscle (BERGQUIST and DEUTSCH 1953) as well as in invertebrate muscle (NILSSON 1957). However, the content of adenine nucleotides in rat diaphragm is approximately one half of the content in skeletal muscle, but twice as high as in invertebrate muscle. It should be mentioned that FLECKENSTEIN and JANKE (1953) and FLECKENSTEIN et al. (1954), in frog skeletal muscle and also in rat diaphragm (FLECKENSTEIN 1955), identified ATP, ADP and in addition a nucleotide called "Die dritte Fraktion". This last mentioned fraction which was demonstrated by the technique of paper chromatography, may according to the present work be DPN.

By the chromatographic fractionation of extracts from rat diaphragm and extracts of rat skeletal muscle two complexes of ADP have been isolated but not fully identified. Indications have been obtained that these components may be similar to a breakdown product from DPNH formed during the fractionation procedure. A similar compound has been observed by chromatographic separation of acid soluble nucleotides from liver (HURLBERT et al. 1954), but not from brain or tumor tissue (SCHMITZ et al. 1954). Isolation of a nucleotide from liver identified as ADP-ribose has been reported by HANSEN et al. (1956).

Rat diaphragm initially contained relatively large amounts of

IMP plus inosine (25—30 per cent). Apparently this reflects the over all conversion $ATP \rightarrow IMP \rightarrow$ Inosine during postmortem twitchings. The breakdown of ATP to inosinic acid in muscle contraction is known from the classical experiments of PARNAS (1929) and has also been demonstrated in isolated muscle fibres during a single twitch (WAJZER et al. 1956).

In incubation experiments a breakdown of ATP and formation of IMP plus inosine is characteristic. Under aerobic conditions the level of ATP is slightly reduced, but in anaerobiosis or by addition of 2,4-DNP the breakdown of ATP during incubation is greatly accelerated. Probably the sequence of reactions $ATP \rightarrow ADP \rightarrow AMP \rightarrow IMP \rightarrow$ inosine occurs as a result of the combined action of ATPase, myokinase, 5'adenylic acid deaminase and 5'nucleotidase. The breakdown does not proceed beyond the stage of inosine and only trace quantities of hypoxanthine, xanthine and uric acid are formed. This shows that nucleoside phosphorylase activity is very low in the surviving rat diaphragm.

It must be expected that in rat diaphragm kept under anaerobic conditions with the very low level of ATP observed, several energy requiring reactions will suffer. One consequence of the anaerobic condition, namely the inability to maintain insulin effect on glucose uptake will be discussed in investigations to be published.

Summary.

1. The content of acid soluble mononucleotides in the rat diaphragm has been determined during incubation under aerobic and anaerobic conditions and after addition of 2,4-dinitrophenol. The diaphragms were incubated in Krebs Ringer phosphate medium, pH 7.4 and 37° C, and the mononucleotides determined in PCA extracts of the diaphragms subjected to chromatography on Dowex-1 anion exchange resin.

2. Eleven different nucleotide components were obtained from rat diaphragm. The bulk was composed of adenine nucleotides, ATP representing 50 per cent of the total content. Small amounts of GTP and UTP were present. Two complexes of ADP have been detected but not fully identified. Incorporation of P^{32} from radioactive inorganic phosphate into these two last mentioned compounds was small after 30 minutes incubation.

3. By aerobic incubation for 30 minutes the level of ATP was

decreased to slightly below the level initially present. Addition of insulin or adrenalin *in vitro* had no effect on the content of ATP in the diaphragm during incubation.

4. In anaerobiosis or by addition of NaCN or 2,4-DNP to the medium, ATP in rat diaphragm was rapidly broken down. After incubation for 30 minutes the content of ATP was decreased to a level of 10 per cent of the amount initially present. Simultaneously, formation of IMP and inosine occurred in amounts corresponding to the breakdown of ATP. A great deal of inosine was liberated into the medium during incubation. The investigations suggest that during incubation of rat diaphragm ATP is broken down by the subsequent action of ATPase, myokinase, 5'adenylic acid deaminase and 5'nucleotidase.

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Institute of Medical Biochemistry and Physiology Department of
Biochemistry, University of Oslo, Norway.

Enzymic Conversion of Mononucleotides by Rat Diaphragm *in vitro*.

By

AA. RYE ALERTSEN, O. WALAAS and E. WALAAS.

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Evidence has been presented that insulin stimulates uptake of hexoses and pentoses in muscle by facilitating the transport of sugars across the surface membrane (LEVINE and GOLDSTEIN 1955). In this connection studies of the structure and metabolic activities of cellular surface membranes are of considerable interest. In experiments by ROTHSTEIN and MEIER (1948, 1949) on living yeast cells it has been shown that phosphatases and ATPases are localized at the cell surface. Further, evidence has been obtained (ROTHSTEIN 1956) that sugar uptake in the yeast cell is due to enzyme activity at the cell surface.

Abbreviations used.

AMP: Adenosine-5'-monophosphate
ADP: Adenosine diphosphate.
ATP: Adenosine triphosphate.
IMP: Inosine-5'-monophosphate.
IDP: Inosine diphosphate.
ITP: Inosine triphosphate.
GMP: Guanosine-5'-monophosphate.
GDP: Guanosine diphosphate.

GTP: Guanosine triphosphate.
CMP: Cytidine-5'-monophosphate.
CDP: Cytidine diphosphate.
CTP: Cytidine triphosphate.
UMP: Uridine-5'-monophosphate.
UDP: Uridine diphosphate.
UTP: Uridine triphosphate.

In the isolated rat diaphragm, ATPase activity, probably located at the cell surface, has been demonstrated by MARSH and HAUGAARD (1957). ZIERLER, LEVY and ANDRES (1953) have reported that leakage of glycolytic enzymes from rat diaphragm occurs during incubation. This is particularly pronounced with aldolase (ZIERLER 1956). Further, it has been shown by ZIERLER (1957) that the efflux of aldolase was increased by addition of insulin to the incubation medium.

In the present work the presence of dephosphorylating as well as transphosphorylating enzymic activity has been demonstrated by incubation of the isolated rat diaphragm *in vitro*. It has been shown that ATPase activity is firmly bound to the muscle, while myokinase, 5'adenylic acid deaminase and nucleoside diphosphokinase are easily liberated into the medium during incubation. These results have been obtained by adding several nucleotides to the incubation medium of rat diaphragm, and fractionation of the conversion products by chromatography on Dowex-1 anion exchange resin.

Material and Methods.

In each experiment two hemidiaphragms from grown rats weighing 250 g were used. The rat was decapitated, bled and after removal the hemidiaphragms were kept in ice cold Krebs Ringer phosphate medium for ten minutes. The muscle was trimmed with scissors so that the total weight of the two hemidiaphragms was about 300 mg. The muscle tissue was then blotted gently on filter paper and transferred to 10 ml Erlenmeyer vessels containing 2 ml Krebs Ringer phosphate medium pH 7.4 to which was added $10 \mu\text{M}$ of nucleotide. Incubation was done in a Warburg bath at 37°C , usually for 15 to 30 minutes. In aerobic experiments the medium was equilibrated with 100 per cent oxygen. In anaerobic experiments argon was used as the gase phase.

When incubation was finished the diaphragms were removed and rinsed twice with 1 ml Krebs Ringer phosphate medium. The medium and the washing solutions were combined and precipitated with 2 ml 0.6 N PCA. Preparation of an extract for chromatography by the method of HURLBERT et al. (1954), and identification of the different fractions obtained was done by a procedure described in a preceding paper (ALERTSEN, WALAAS and WALAAS 1958).

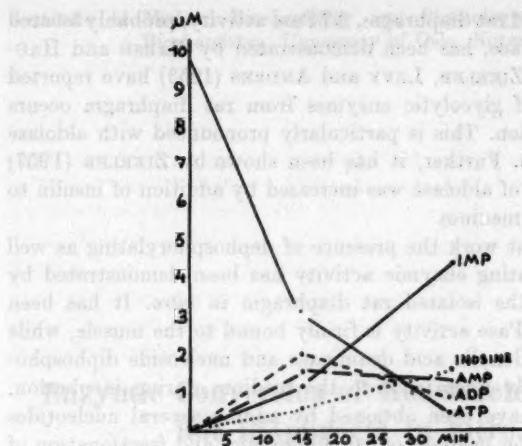


Fig. 1. Breakdown of ATP by the rat diaphragm *in vitro*. Krebs Ringer phosphate medium pH: 7.4, 100 per cent oxygen. Initially 10 μ M ATP was added to the medium.

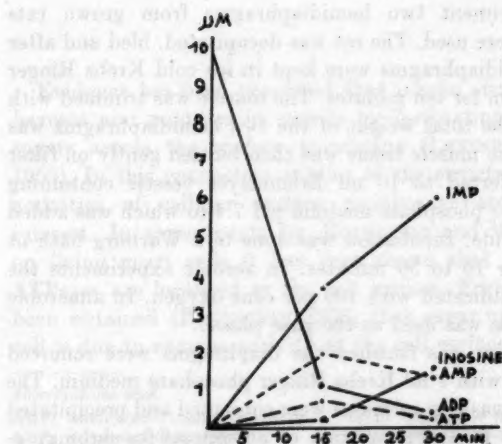


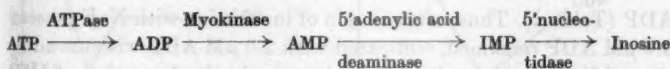
Fig. 2. Conversion of ADP by the rat diaphragm *in vitro*. Krebs Ringer phosphate medium pH: 7.4, 100 per cent oxygen. Initially 10 μ M ADP added to the medium.

The nucleotides and nucleosides used in these experiments were obtained from "Sigma" Chemical Company, St. Louis, except ADP and GTP which came from Nutritional Biochemical Corporation, Cleveland. Before use the products were purified by the chromatographic procedure. This was done because the preparations of nucleotides were contaminated with other nucleotides to some extent, as illustrated below.

Compound	Contaminations
10 μ M ATP	below 0.1 μ M ADP
10 μ M ADP	1.0 μ M AMP + 0.4 μ M ATP + 0.6 μ M ADP-X
10 μ M UTP	1.0 μ M UDP
10 μ M ITP	0.5 μ M IDP

Results.

As shown in Fig. 1 rat diaphragm exerts a pronounced ATPase activity *in vitro*. After 15 minutes of incubation the ATP added to the medium was broken down to a great extent and ADP plus AMP were formed. The breakdown of ATP was slightly decreased if Mg^{++} was omitted from the medium. However, the breakdown was of the same order of magnitude by incubation under aerobic or anaerobic conditions. By incubation for 30 minutes the adenine nucleotides declined to very low levels. Simultaneously IMP and inosine were formed in considerable amounts. This experiment indicates that ATP is broken down by the following sequence of enzymatic reactions:



In further experiments evidence for the presence of these enzymic activities has been obtained. As shown in Fig. 2 myokinase activity has been demonstrated. By addition of 10 μ M ADP to the incubation medium 0.7 M ATP was synthesized by incubation for 15 min. However, the ATP which is formed was rapidly broken down due to ATPase activity of the diaphragm and after 30 min most of the ADP was converted to AMP, IMP and inosine. Addition of 0.02 M NaF, which is known as an inhibitor of myokinase, (SIEKEVITZ and POTTER 1953) partly prevented the conversion of

Table I.

Inhibition of myokinase activity of rat diaphragm by NaF. Krebs Ringer phosphate medium pH: 7.4. Incubation for 15 min in 100 per cent oxygen. The medium contained 0.02 M NaF. 10 μ M ATP was added initially.

Component	Recovered after incubation		Effect of NaF μ M
	With NaF μ M	Without NaF μ M	
ATP	3.2	3.1	+ 0.1
ADP	3.0	2.0	+ 1.0
AMP	1.8	1.6	+ 0.2
IMP	0.5	1.2	- 0.7
Inosine	Trace	0.6	- 0.6

Table II.

Conversion of AMP by the rat diaphragm in vitro. Krebs Ringer phosphate medium pH: 7.4. 15 min incubation. 10 μ M AMP added initially to the medium.

Component	Recovery after incubation μ M
AMP	0.9
IMP	6.6
Inosine	1.3

ADP (Table I). Thus, after 15 min of incubation with NaF present 3.0 μ M ADP remained, compared with 2.0 μ M ADP without addition of NaF to the medium. Simultaneously the formation of IMP and Inosine was decreased.

5'adenylic acid deaminase activity was demonstrated by incubation of rat diaphragm with 5'adenylic acid in the medium. As shown in Table II a rapid conversion to inosinic acid took place. Further, a small amount of inosine was formed apparently by 5'nucleotidase activity. The possibility that inosine was formed by the sequence of reactions AMP \rightarrow Adenosine \rightarrow Inosine was excluded. Thus, by incubation of rat diaphragm with adenosine no formation of inosine was detected.

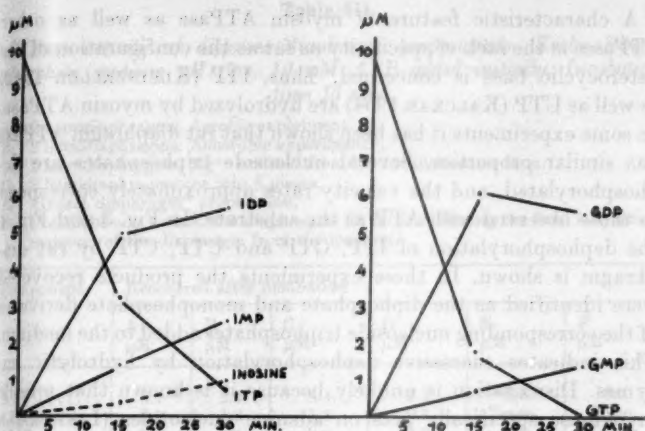


Fig. 3. Breakdown of ITP and GTP by the rat diaphragm *in vitro*.
 Krebs Ringer phosphate medium pH: 7.4.

A: 10 μM ITP added initially.

B: 10 μM GTP added initially.

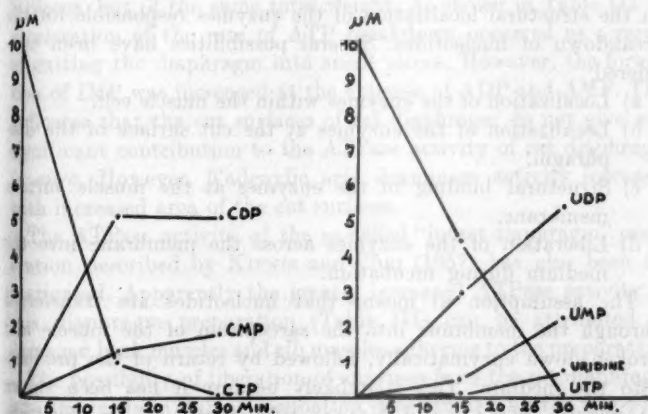


Fig. 4. Breakdown of CTP and UTP by the rat diaphragm *in vitro*.
 Krebs Ringer phosphate medium pH: 7.4.

A: 10 μM CTP added initially.

B: 10 μM UTP added initially.

A characteristic feature of myosin ATPase as well as other ATPases is the lack of specificity as far as the configuration of the heterocyclic base is concerned. Thus, ITP (KLEINZELLER 1942) as well as UTP (KALCKAR 1954) are hydrolyzed by myosin ATPase. In some experiments it has been shown that rat diaphragm ATPase has similar properties. Several nucleoside triphosphates are dephosphorylated, and the velocity rates approximately correspond to those observed with ATP as the substrate. In Fig. 3 and Fig. 4 the dephosphorylation of ITP, GTP and UTP, CTP by rat diaphragm is shown. In these experiments the products recovered were identified as the diphosphate and monophosphate derivatives of the corresponding nucleoside triphosphates added to the medium. This indicates successive dephosphorylation by hydrolytic enzymes. Dismutation is unlikely because it is known that muscle myokinase specifically acts on adenine nucleotides (LIEBERMAN et al. 1954). Any further conversion to nucleosides has only been observed to a very small extent. No evidence was obtained of deamination of guanosine or cytidine nucleotides. In these experiments the recovery was nearly quantitative, *i. e.* above 80 per cent of the nucleotides added could be accounted for after incubation.

Some experiments were performed in order to get information on the structural localization of the enzymes responsible for the breakdown of nucleotides. Several possibilities have been considered:

- a) Localization of the enzymes within the muscle cell.
- b) Localization of the enzymes at the cut surface of the diaphragm.
- c) Structural binding of the enzymes at the muscle surface membrane.
- d) Liberation of the enzymes across the membrane into the medium during incubation.

The assumption a) means that nucleotides are transferred through the membrane into the sarcoplasm of the muscle and broken down enzymatically, followed by return of the products into the medium. This is unlikely because it has been shown (ALERTSEN, WALAAS and WALAAS 1958) that ATP preexisting in the diaphragm is only broken down to a small extent by aerobic incubation. Obviously, ATPase activity within the diaphragm which is pronounced under anaerobic conditions, is counteracted by oxidative phosphorylations.

Table III.

ATPase activity of different diaphragm preparations. Krebs Ringer phosphate medium pH: 7.4. 10 μ M ATP added initially. Incubation time 15 min.

- A: 2 hemidiaphragms. Aerobic experiment.
 B: 2 hemidiaphragms. Anaerobic experiment.
 C: 2 hemidiaphragms. Mg^{++} omitted from the incubation medium.
 D: 2 hemidiaphragms cut into 8 pieces.
 E: "Intact diaphragm" preparation.
 F: Preincubation with 2 hemidiaphragms for 30 min. During the final incubation omission of the diaphragm from the medium.

Component	Recovered after incubation					
	A μ M	B μ M	C μ M	D μ M	E μ M	F μ M
ATP	3.1	2.5	4.4	2.2	0.4	8.5
ADP	2.0	1.8	1.8	0.7	Trace	Trace
AMP	1.6	1.5	0.2	0.3	0.1	Trace
IMP	1.2	2.7	1.1	5.1	5.9	Trace
Inosine	0.6	0.6	Trace	0.7	2.0	Trace

The contribution of cut surfaces of the diaphragm to enzyme activity, has been studied by comparing ATPase activity of two hemidiaphragms with that of hemidiaphragms cut into a total of 8 pieces, but of the same total weight. As shown in Table III no acceleration of the rate of ATP breakdown occurred as a result of cutting the diaphragm into small pieces. However, the formation of IMP was increased at the expense of ADP and AMP. This indicates that the cut surfaces of rat diaphragm do not give any significant contribution to the ATPase activity of rat diaphragm *in vitro*. However, 5'adenylic acid deaminase activity increases with increased area of the cut surfaces.

The ATPase activity of the so-called "intact diaphragm" preparation described by KIPNIS and CORI (1957) has also been investigated. Apparently the greatly increased ATPase activity of this diaphragm preparation (Table III) can be attributed to damaged back muscles and rib muscles adhering to the preparation.

The possibility of liberation of enzymes from the rat diaphragm into the medium during incubation, was studied by the following procedure. Two hemidiaphragms were incubated in 2 ml medium by the usual technique, but without addition of nucleotide to the medium. After 30 minutes of incubation at 37° C the hemidiaphragms were removed from the vessel. 10 μ M ATP was then

Table IV.

Leakage of myokinase and 5'adenylic acid deaminase from the rat diaphragm into the medium.

Krebs Ringer phosphate medium pH: 7.4. Preincubation with 2 hemidiaphragms for 30 min. At the final incubation period of 30 or 15 min ADP or AMP was added and the diaphragms were omitted from the medium.

Component	Recovery after incubation	
	10 μ M ADP added 30 min incubation μ M	10 μ M AMP added 15 min incubation μ M
ATP	1.6	
ADP	0.7	
AMP	0.1	2.9
IMP	4.9	6.2
Inosine	Trace	0.3

added and incubation continued for another 30 min. As shown in Table III no significant ATPase activity could be demonstrated because only trace quantities of ADP, AMP and IMP were formed and 85 per cent of ATP was recovered unchanged. In similar experiments ADP and AMP were used as substrates instead of ATP. In Table IV it is clearly shown that unlike ATPase, myokinase as well as 5'adenylic acid deaminase is easily liberated into the medium during incubation.

In conclusion, these experiments have shown that ATPase activity of the rat diaphragm is a property of the surviving muscle, probably localized at the surface membrane structures.

Nucleoside diphosphokinase ("nudiki") activity has also been demonstrated by incubation of rat diaphragm *in vitro*. These enzymes are liberated into the medium as shown in experiments presented in Table V. Two hemidiaphragms were preincubated for 30 min at 37° C and the muscle tissue was then removed to avoid any ATPase activity. Thereupon 9 μ M each of ATP and IDP were added to the medium. The synthesis of 5.1 μ M of ITP clearly shows the presence of nucleoside diphosphokinase, according to the reaction: $ATP + IDP \rightleftharpoons ADP + ITP$. The small recovery of 1.7 μ M ADP is explained by the breakdown $ADP \rightarrow AMP \rightarrow IMP$ due to myokinase and 5'adenylic acid deaminase liberated into the medium.

In corresponding experiments where ATP and UDP were added to the medium, UTP was synthesized according to the reaction:

Table V.

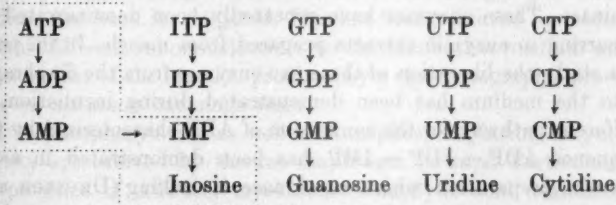
Liberation of nucleoside diphosphokinase from the rat diaphragm into the medium during incubation. Procedure as mentioned in Table IV.

9 μ M each of ATP and IDP added to the medium during the final incubation period of 15 min.		10 μ M each of ATP and UDP added to the medium during the final incubation of 15 min.	
Component	Change during incubation μ M	Component	Change during incubation μ M
ATP	- 4.5	ADP	+ 1.9
ADP	+ 1.7	AMP	+ 0.3
AMP	+ 0.3	IMP	+ 1.5
ITP	+ 5.1	UTP	+ 5.0
IDP	- 7.0		
IMP	+ 2.3		

ATP + UDP \rightleftharpoons ADP + UTP. UTP formed in this experiment appeared as a chromatographic peak with localization different from the commercially obtained UTP. However, after lyophilization the compound was identified as uridintriphosphate because of its u. v. spectrum and its content of phosphorus.

Discussion.

The enzymatic breakdown of nucleoside triphosphate added to the medium during incubation of rat diaphragm occurs by the sequence of reactions presented in the scheme below.



Of the possible pathways indications have been obtained that ATP is broken down stepwise by the route inside the stippled line. The preceding work (ALERTSEN, WALAAS and WALAAS 1958) has provided evidence that ATP existing within the diaphragm is broken down by the same sequence of reactions. A similar pathway for the breakdown of ATP by other animal

tissues has been reported. In brain homogenate ATP is broken down to inosine with ADP, AMP and IMP occurring as intermediate products (SMILLIE 1957) and in red cells the breakdown proceeds to the stage of hypoxanthin (CHEN and JØRGENSEN 1956). In embryonic tissue nucleoside triphosphate are rapidly converted to the corresponding diphosphate derivatives in anaerobic incubation experiments (TIEDEMANN 1957).

Evidence is presented that the ATPase activity is associated with the membrane surface of the diaphragm, and no liberation of ATPase into the medium has been demonstrated. Thus, in addition to myosin ATPase (ENGELHARDT and LJUBIMOVA 1939) and sarcosome ATPase (BRIGHAM, BRINCH-JOHNSEN and WALAAS 1956), ATPase activity is localized at the membrane surface of rat diaphragm. This is in accordance with the findings of MARSH and HAUGAARD (1957).

The breakdown of GTP, ITP, UTP and CTP to the diphosphate stage by the isolated rat diaphragm can be attributed to the non-specificity of ATPase. It is known that myosin ATPase and actomyosin gels dephosphorylate GTP, ITP and UTP in addition to ATP (KALCKAR 1954, BERGQUIST and DEUTSCH 1954, BLUM 1955). The splitting of UTP by myosin ATPase is even faster than the breakdown of ATP. The rate of breakdown of GTP by the diaphragm was somewhat faster than the splitting of the other nucleoside triphosphates, but no detailed study of this problem has been made.

The conversion of ADP to IMP in muscle can be attributed to the action of two enzymes: (1) Dismutation to AMP + ATP by myokinase, and (2) Deamination of AMP by 5'adenylic acid deaminase. These enzymes have repeatedly been demonstrated as occurring in enzymic extracts prepared from muscle. In the present study the liberation of these two enzymes from the diaphragm into the medium has been demonstrated during incubation. A different pathway for the conversion of ADP characterized by the sequence $ADP \rightarrow IDP \rightarrow IMP$ has been demonstrated in actomyosin preparation where myokinase is lacking (DEUTSCH and NILSSON 1954), in washed myofibrils (WEBSTER 1953) and in water soluble extracts of skeletal muscle (DEUTSCH and NILSSON 1954). The deamination of ADP is reported to be a slow reaction and no indication has been obtained that it takes place in the rat diaphragm during incubation *in vitro*. Thus, IDP was never recovered among the reaction products when incubation of the diaphragm was performed with addition of ATP or ADP.

A leakage of nucleoside diphosphokinase into the medium has also been demonstrated by incubation of rat diaphragm. Observations have been made that such enzymes are widely distributed in muscle (KREBS and HEMS 1953, KALCKAR 1953).

In conclusion, these experiments have shown that several dephosphorylating and transphosphorylating enzymic activities are exerted by the rat diaphragm during incubation *in vitro*. Evidence is presented that ATPase activity is firmly associated with the surface membrane of the diaphragm, while myokinase, 5'adenylic acid deaminase and nucleoside diphosphokinase are liberated from the muscle into the medium. The difference must be attributed either to a difference structural binding of the enzymes in the muscle or to specificity of penetration of enzyme molecules across muscle surface membrane. Similar specificity is known from experiments on efflux of glycolytic enzymes, the liberation of aldolase being particularly pronounced (ZIERLER 1956). The conversion of glucose to lactic acid by the diaphragm could partly be attributed to an enzyme system which appeared to be at or near the cell surface (SHAW and STADIE 1957) but which was not liberated into the medium. By incubation of rat diaphragm no leakage of hexokinase into the medium could be demonstrated in the investigation by WALAAS (1955).

Summary.

1. The enzymatic conversion of mononucleotides by the isolated rat diaphragm during incubation in Krebs Ringer phosphate medium has been investigated. This has been achieved by adding several different mononucleotides to the incubation medium and fractionation of the reaction products by a chromatographic procedure on an anion exchange resin.

2. Dephosphorylating as well as transphosphorylating enzymic activity are exerted by the rat diaphragm *in vitro*. Particularly the presence of ATPase, myokinase, 5'adenylic acid deaminase and nucleoside diphosphokinase has been demonstrated.

3. The breakdown of ATP added to the medium proceeds by the sequence $ATP \rightarrow ADP \rightarrow AMP \rightarrow IMP \rightarrow \text{Inosine}$. By addition of ITP, GTP, CTP, and UTP, these nucleoside triphosphates are broken down to the corresponding diphosphate and monophosphate derivatives.

4. Indications have been obtained that ATPase is firmly bound

at or near the muscle cell surface. On the other hand a leakage of myokinase, 5'adenylic acid deaminase and nucleoside diphosphokinase from the diaphragm into the medium during incubation has been demonstrated.

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From the Department of Pharmacology, Karolinska Institutet, Stockholm 60, Sweden.

The Release of Catechols from the Adrenal Medulla on Activation of the Sympathetic Vasodilator Nerves to the Skeletal Muscles in the Cat by Hypothalamic Stimulation.

By

R. GRANT¹, P. LINDGREN, A. ROSEN and B. UVNÄS.

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Since the original reports of HOUSSAY and MOLINELLI (1925) and MAGOUN, RANSON and HETHERINGTON (1937), many investigators have observed a discharge of catechols from the adrenal medulla on hypothalamic stimulation as well as on stimulation in the mesencephalon (LINDGREN 1955) and the oblongate medulla (CHEN *et al.* 1936, 1937 a and b).

Considerable interest attaches to the recent reports that hypothalamic stimulation may produce a selective increase of the adrenaline outflow (BRÜCKE, KAINDL and MAYER 1952), and that stimulation at various sites in that region produces sometimes chiefly a noradrenaline outflow, and sometimes chiefly an adrenaline outflow (REDGATE and GELLHORN 1953, FOLKOW and EULER 1954).

¹ Present address: Stanford University, Stanford, California, U. S. A.

In a series of investigations conducted by UVNÄS and co-workers it was shown that the sympathetic vasodilator nerves to the skeletal muscles in dogs and cats could be activated by intracerebral stimulation at various levels (for references, see UVNÄS (1954)). If care was taken to activate the sympathetic vasodilator outflow by stimulation of low or moderate intensity — in order to avoid pressor responses — the vasodilatation in the skeletal muscles was in most cases completely abolished by atropinization of the animal. If, however, the vasodilator responses were accompanied by pressor responses, the increase of blood flow in the skeletal muscles was only partially inhibited by atropine. Since the increase remaining after atropinization often seemed greater than was to be expected, if it were due only to the hemodynamic effect of the elevated blood pressure, part of it might well have been attributable to the vasodilator action of adrenaline released from the adrenals. In fact, LINDGREN and UVNÄS (unpublished observations) and LINDGREN (1955) had observed that after unilateral sympathectomy, vasomotor responses still occurred in the sympathectomized leg on activation of the sympathetic vasodilator outflow to the contralateral leg by stimulation at hypothalamic and mesencephalic levels. In both hind legs vasodilatation appeared in the skeletal muscles and vasoconstriction in the skin, but both types of vasomotor response occurred with an appreciably longer latency in the sympathectomized leg. The occurrence of delayed vascular responses in the sympathectomized leg indicated release of catechols from the adrenals. Since it seemed of interest to obtain further information on the amounts of catechols secreted concomitant with activation of the sympathetic vasodilator nerves by intracerebral stimulation, a series of quantitative determinations was made of adrenaline and noradrenaline released into the adrenal vein during such vasodilator activation.

Methods.

Complete experiments were performed on 14 cats (weighing 2.5–4.5 kg) under Dial (50 mg/kg), urethane (800–1,200 mg/kg) or a combination of chloralose (50 mg/kg) and urethane (300 mg/kg). Topical stimulation in the brain was effected in 12 cats with a unipolar and in 2 cats with a bipolar electrode, oriented by the Horsley-Clarke technique. A stimulator yielding square-wave impulses with an output resistance of 1,000 ohms was used. The duration of each impulse was 2 msec, the

frequency usually 70/sec. For stimulation in the hypothalamus the electrode was inserted through a hole made in the roof of the skull.

For recording the blood flow from a skeletal muscle region, the femoral or popliteal vein was cannulated and the blood directed to a photo-tube drop-counter operating an ordinate recorder. The blood was then allowed to re-enter the animal via the cannulated proximal stump of the same vein (LINDGREN and UVNÄS 1954). The leg was skinned and the circulation of the paw shut off by a tight ligature just above the ankle. To prevent clotting, heparin was given intravenously (25 mg/kg).

The blood pressure was measured in a carotid artery with a mercury manometer.

Histologic examination was performed on 12 brains. The brains were fixed in 10 per cent formaldehyde solution by intracarotid injection in situ of 40–60 ml. Frozen serial sections were made, 50 μ thick, in a plane parallel to the electrode puncture. Every fourth section was stained with Weil's hematoxylin method.

Catechol Assay.

Blood was collected from the left adrenal vein during hypothalamic stimulation and during control periods between the stimuli. Usually 3–6 ml were obtained in about 3–5 minutes. The blood was collected in ice cooled siliconed glass tubes and centrifuged, and the plasma taken for catechol assay.

The loss of fluid due to the repeated sampling of blood was compensated for by injecting intravenously equivalent volumes of dextran (Macrodex, Pharmacia). Between collecting periods the venous outflow from the adrenal was continuously recorded by the method described above and the blood was allowed to re-enter the animal via the jugular vein.

The catechol content of the adrenal blood was assayed in two ways.

1. Direct testing of the plasma.
2. Testing after separating adrenaline and noradrenaline by paper chromatography.

In both cases the biologic assay was performed on rat uterus and rat colon with the procedure reported by GADDUM and LEMBECK (1949).

For paper chromatography the plasma was treated, in principle, according to VOGT (1952). The procedure involved (1) extraction with ethanol; (2) extraction with NaCl-saturated ethanol to reduce the content of potassium salts (BARSOUM and GADDUM 1935); and (3) extraction with acetone and ethanol to reduce the content of inorganic substances and of organic compounds insoluble in acetone.

The chromatography procedure was *ad modum* CRAWFORD and OUTSCHORN (1950) with the modifications of VOGT (1952).

For development of the chromatograms a mixture of phenol with 15 per cent 0.1 N HCl was used. The jars were filled with CO₂ and the chromatograms were run for 18–20 hours at room temperature. Elution was done with 12 ml phosphate solution overnight. The eluate was concentrated over a water bath *in vacuo* and the dry residue dis-

solved in ascorbic acid solution (50 $\mu\text{g}/\text{ml}$) just before the biologic assay.

Even though the adrenal blood catechol values obtained by the direct and indirect methods showed, on the whole, fairly close agreement, all values given in this paper refer to the indirect method. Notwithstanding the risk of catechol losses in the rather elaborate extraction procedure necessitated by the chromatography method, our experience is that this method yielded less varying results. Direct testing of the plasma sometimes gave higher, sometimes lower values than the indirect method — a fact which suggested the presence in untreated plasma of substances interfering with the biologic assay of catechols, as was also pointed out by Voegt (1952).

The loss of adrenaline and noradrenaline during the paper chromatography procedure was determined by adding known amounts of catechols to adrenal blood plasma. The yield amounted to 60–100 per cent for both adrenaline and noradrenaline. (The figures in the present paper have not been corrected for this loss.)

Results.

Hypothalamic Stimulation.

Topical stimulation in the hypothalamus elicited, in the hind leg muscles, vasodilator responses of the same character as those described in several previous papers from our laboratory. The blocking action of atropine was taken as evidence that the vasodilatation resulted from activation of the sympathetic cholinergic vasodilator outflow. In 14 cats the concomitant output of catechols from the left adrenal was determined. In all, we were able to record the catechol output during 29 hypothalamic stimulations, with satisfactory control samples from the adrenal outflow before and after the stimulation periods.

Fig. 1 illustrates a typical experiment. The vasodilator outflow was activated both by repeated volleys of stimuli — 3×15 sec for 3 min (1–3 in Fig. 1) — and by continuous stimulation for 3 min (4 in Fig. 1). The stimulation caused a three- to fourfold increase of the limb blood flow.

Together with the vasodilator effects we observed an increase in the catechol output (Fig. 2). This was especially the case with the adrenaline discharge, which increased by 200 (sample A) and 100 per cent (sample C), respectively. The noradrenaline, on the other hand, increased by only 23 and decreased by 7 per cent, respectively. (The exact figures are reported in Table I, cat no. 9.) As a control that the vasodilator effects were due to activation of sym-

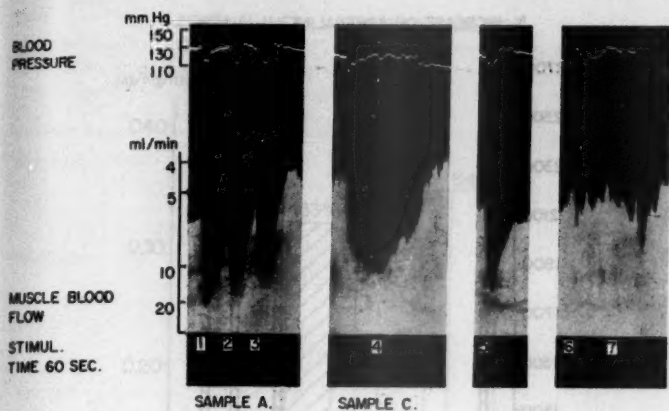


Fig. 1. Cat no. 9, 3.1 kg. Urethane 860 mg/kg. Vasodilator responses in the muscles of the right hind leg to stimulation in the hypothalamus.

Samples A and C indicate the catechol output seen in Fig. 2. 1, 2, 3, 5. Stimulation, 2 V, 70 imp/sec. 4. Stimulation, 1.5–2 V, 20 imp/sec. Between 5 and 6: Atropine 0.1 mg/kg i. v. 6. Stimulation, 2 V, 70 imp/sec. 7. Stimulation, 1.5–2 V, 20 imp/sec.

Note. The vasodilator responses are abolished after atropinization.

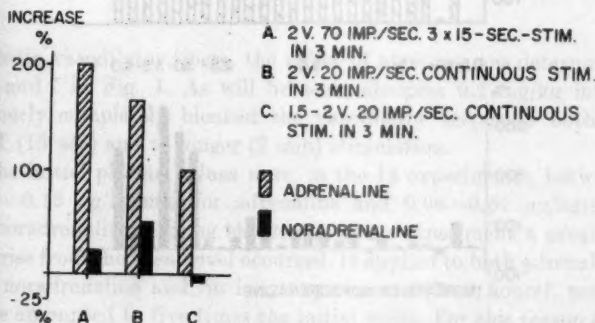


Fig. 2. Catechol output from the left adrenal gland on activation of the sympathetic vasodilator outflow by hypothalamic stimulation (the same experiment as illustrated in Fig. 1). The percentual increase is calculated from the control level recorded 5 min before each stimulation.

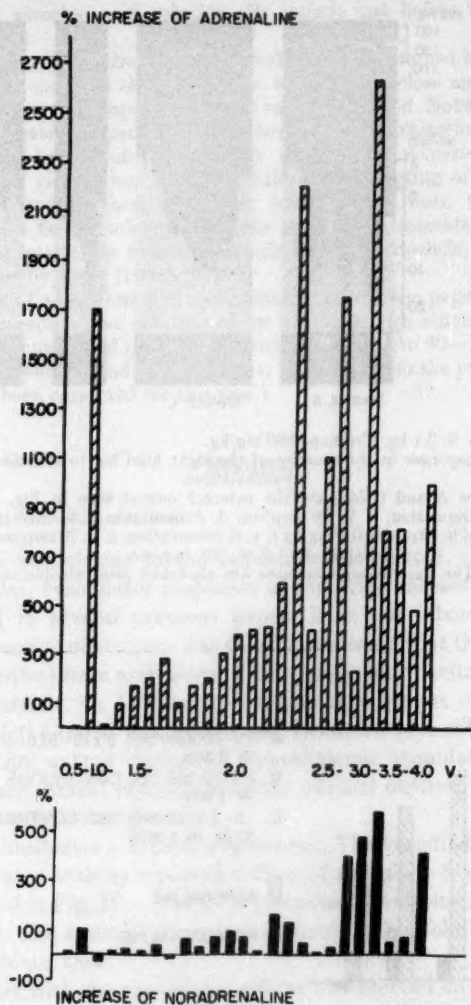


Fig. 3. Relative values from 25 stimulations. Survey of the results from 12 experiments on cats anesthetized with urethane or chloralose-urethane. Catechol output from one adrenal gland on activation of the sympathetic vasodilator outflow by hypothalamic stimulation. Per cent increase is calculated from the control level taken 5 min before each stimulation.

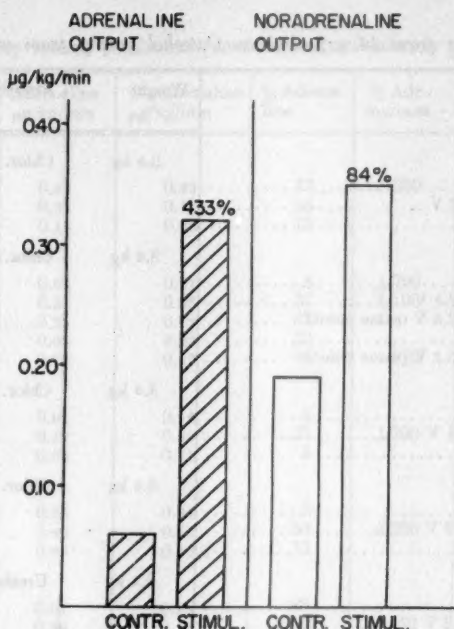


Fig. 4. Mean values of the adrenaline and noradrenaline output, control level and during hypothalamic stimulation (25 control and stimulation tests).

pathetic vasodilator fibers, the effect of atropine was determined at 6 and 7 in Fig. 1. As will be seen, atropine 0.1 mg/kg intravenously completely blocked the vasodilator responses both to brief (15 sec) and to longer (2 min) stimulation.

The initial plasma values were, in the 14 experiments, between 0.01–0.12 $\mu\text{g/kg/min}$ for adrenaline and 0.06–0.67 $\mu\text{g/kg/min}$ for noradrenaline. During the course of an experiment a progressive rise from the basal level occurred. It applied to both adrenaline and noradrenaline and, in long experiments (3–4 hours), sometimes amounted to five times the initial value. For this reason the catechol content of the adrenal outflow was determined immediately before each stimulation, and the stimulatory increase of the catechol output was calculated, as a rule, from the prestimulatory level. The stimuli were not given at shorter intervals than 15 minutes.

Table

Results from 14 experiments. Catechol output from one adrenal

	Weight	Anesthesia
<i>Cat no. 1:</i>	3.5 kg	Chlor. + urethane
Control		
Stimulation 2 V		
Control		
<i>Cat no. 2:</i>	3.2 kg	Chlor. + urethane
Control		
Stimulation 2.5 V		
Stimulation 2.5 V (same point)		
Control		
Stimulation 3.5 V (same point)		
<i>Cat no. 3:</i>	4.5 kg	Chlor. + urethane
Control		
Stimulation 1 V		
Control		
<i>Cat no. 4:</i>	3.5 kg	Chlor. + urethane
Control		
Stimulation 2 V		
Control		
<i>Cat no. 5:</i>	2.5 kg	Urethane
Control		
Stimulation 2 V		
Control		
Stimulation 4 V (same point)		
Control		
Stimulation 4 V (same point)		
Control		
<i>Cat no. 6:</i>	4.0 kg	Urethane
Control		
Stimulation 1.5 V		
Control		
<i>Cat no. 7:</i>	4.0 kg	Urethane
Control		
Stimulation 1.5 V		
Stimulation 3 V (same point)		
Control		
<i>Cat no. 8:</i>	3.0 kg	Urethane
Control		
Stimulation 1.5 V		
Control		
<i>Cat no. 9:</i>	3.1 kg	Urethane
Control		
Stimulation 2 V		
Control		
Stimulation 2 V 20/s (same point)		
Control		
Stimulation 1.5—2 V 20/s (same point) ...		

Table
adrenal

I.

gland during resting conditions (control) and stimulation periods.

Electrode	Adrenaline μg/kg/min	Noradrenaline μg/kg/min	% Adrena- line	% Adr.- increase	% Noradr.- increase
unipolar	0.09	0.43	17	300	65
	0.36	0.71	34		
	0.13	0.86	13		
unipolar	0.02	0.47	4	1,750	34 2
	0.37	0.63	37	1,100	
	0.24	0.48	33	810	
	0.08	0.33	20		
	0.73	0.49	60		
unipolar	0.01	0.16	6	1,700	88
	0.18	0.30	27		
	0.03	0.71	4		
unipolar	0.02	0.21	9	2,200	119
	0.46	0.46	50		
	0.08	0.40	17		
unipolar	0.07	0.18	28	410	0
	0.36	0.18	67		
	0.11	0.19	37	480	70
	0.64	0.32	67		
	0.11	0.14	44	990	410
	1.20	0.72	63		
	0.18	0.31	37		
	unipolar	0.08	0.11	42	13
0.09		0.08	53		
0.05		0.08	38		
unipolar	0.03	0.14	18	100 2,630	7 485
	0.06	0.15	29		
	0.82	0.82	50		
	0.06	0.28	18		
unipolar	0.03	0.67	4	200	10
	0.09	0.74	11		
	0.02	0.38	5		
unipolar	0.01	0.13	7	200	23
	0.03	0.16	16		
	0.03	0.28	10		
	0.08	0.42	16	166	50
	0.03	0.31	9		
	0.06	0.29	17	100	—7

Table 1.

	Weight	Anesthesia
<i>Cat no. 10:</i>	3.5 kg	Urethane
Control		
Stimulation 1.5 V		
Control		
Stimulation 2 V (same point)		
Control		
Stimulation 2 V (same point)		
Control		
Stimulation 2.0—2.75 V 15/s (same point) ..		
<i>Cat no. 11:</i>	3.0 kg	Urethane
Control		
Stimulation 2 V		
Control		
Stimulation 0.5 V (same point)		
<i>Cat no. 12:</i>	3.2 kg	Urethane
Control		
Stimulation 1.5 V 20/s		
Control		
Stimulation 3.5 V 20/s (same point)		
Control		
Stimulation 3 V 20/s (same point)		
<i>Cat no. 13:</i>	2.7 kg	Dial
Control		
Stimulation 2 V		
Control		
Stimulation 2 V (same point)		
Stimulation 2 V (new point)		
Control		
<i>Cat no. 14:</i>	3.5 kg	Dial
Control		
Stimulation 2 V		
Control		

Fig. 3 shows the percentage increase in the adrenaline and noradrenaline contents of the adrenal venous outflow on activation of the sympathetic vasodilator outflow. The adrenaline output increased, in some experiments, by more than 2,000 per cent. In these few cases the basal level was very low, but since identical values were obtained with both the direct and indirect method for determination of adrenaline, we feel justified in calculating the increase as a percentage. In 9 of 25 samples the noradrenaline changed very little, and in the rest of them it showed a slight or moderate increase. In practically all cases the increase of the noradrenaline output was much less than that of adrenaline.

Table 1. (Continued.)

Electrode	Adrenaline $\mu\text{g/kg/min}$	Noradrenaline $\mu\text{g/kg/min}$	% Adrena- line	% Adr.- increase	% Noradr.- increase
unipolar	0.03	0.07	30		
	0.08	0.08	50	167	14
	0.05	0.09	36		
	0.24	0.17	59	380	89
	0.04	0.09	31		
	0.20	0.15	57	400	67
	0.05	0.08	38		
	0.25	0.12	68	400	50
unipolar	0.10	0.10	50		
	0.69	0.25	73	590	150
	0.16	0.11	59		
	0.17	0.12	59	6	9
unipolar	0.12	0.06	67		
	0.44	0.11	80	277	83
	0.09	0.06	60		
	0.81	0.41	66	800	580
	0.09	0.06	60		
	0.30	0.30	50	230	400
bipolar	$\mu\text{g/ml}$	$\mu\text{g/ml}$			
	0.04	2	2		
	0.20	2	11	400	0
	0.02	2	1		
	0.50	1	33	2,400	-50
	1.25	1	55	6,150	-50
bipolar	0.05	1	2		
	0.04	3	1		
	2.0	3	40	4,900	0
	0.30	3	11		

The absolute values for adrenaline and noradrenaline are reported in Table I. The catechol discharge caused by hypothalamic stimulation varied considerably from one experiment to another. The maximum value observed for adrenaline was 0.82 $\mu\text{g/kg/min}$ and for noradrenaline 0.82 $\mu\text{g/kg/min}$. As shown in Fig. 4, the mean values for the adrenaline output in the control samples and those taken during stimulation were 0.06 and 0.32 $\mu\text{g/kg/min}$ respectively. The corresponding figures for noradrenaline were 0.19 and 0.35 $\mu\text{g/kg/min}$.

In a minority of our previous experiments, as mentioned above, atropine had failed to block completely the vasodilatation produced

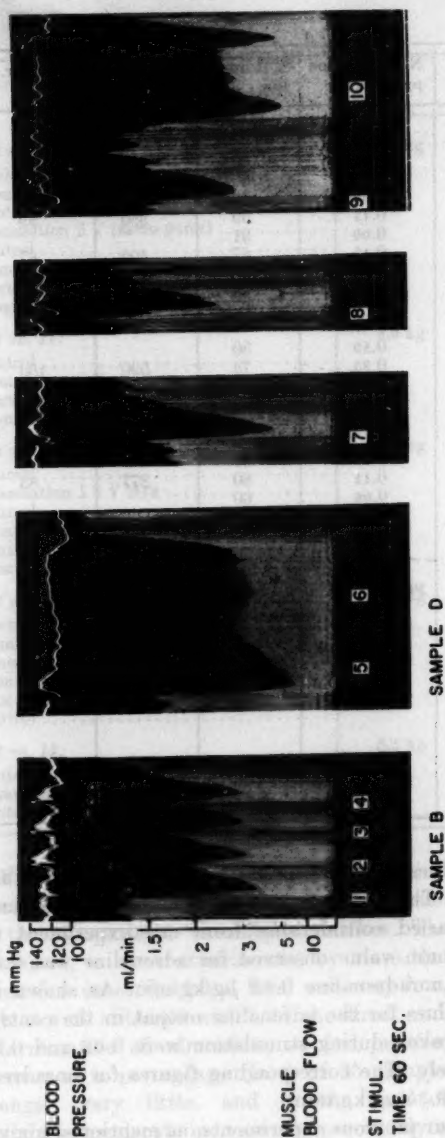


Fig. 5. Cat no. 10, 3.5 kg. Urethane 850 mg/kg. Vasodilator responses in the muscles of the right hind leg to stimulation in the hypothalamus.

1, 2, 3, 4. Stimulation, 2 V, 70 imp/sec.

5. Stimulation, 2 V, 15 imp/sec.

6. Stimulation, 2.75 V, 15 imp/sec.

7. Stimulation, 2 V, 15 imp/sec.

Between 7 and 8: Atropine 0.1 mg/kg i.v.

8, 9, 10. Stimulation, 2 V, 15 imp/sec.

The vasodilator responses are not abolished, only reduced after atropinization. For explanation, see text.

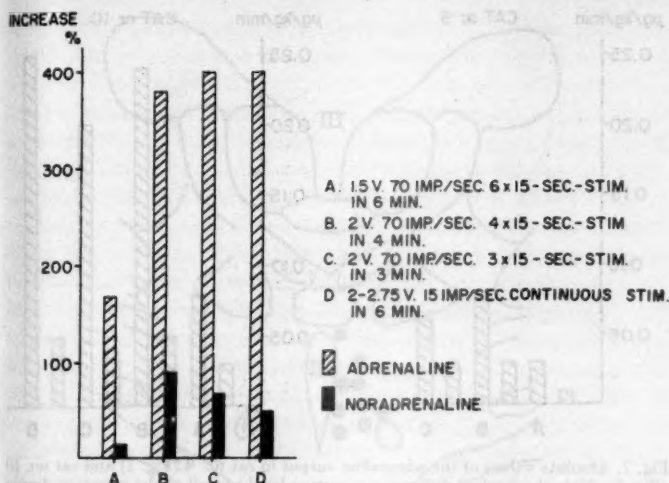


Fig. 6. Catechol output from the left adrenal gland on activation of the sympathetic vasodilator outflow by hypothalamic stimulation (the same experiment as illustrated in Fig. 5). The percentual increase is calculated from the control level recorded 5 min before each stimulation.

in skeletal muscles by hypothalamic stimulation. This observation, it was assumed, might have been due to an output of adrenaline in amounts high enough to have a vasodilator action on the muscle vessels. Fig. 5 and 6 illustrate an experiment in which the vasodilatation was not abolished but only reduced by atropine. The experiment was performed in the same way as that shown in Fig. 1. Especially worthy of note is that the stimulation parameters were essentially identical in the two experiments. In spite of atropinization, pronounced vasodilatation occurred both with brief (15 sec) and with more continuous (3 min) stimulation (9 and 10 in Fig. 5).

In this, as in the rest of the experiments, hypothalamic stimulation induced a discharge of adrenaline into the adrenal venous blood. Of special interest, however, are the absolute values for the catechol output in this experiment compared with that illustrated in Fig. 1. The initial value before hypothalamic stimulation was $0.01 \mu\text{g/kg/min}$ in Fig. 1 and $0.03 \mu\text{g/kg/min}$ in Fig. 5. A direct comparison of the absolute catechol values in the two experiments

Between 7 and 8: Atropine 0.1 mg/kg i.v.
8, 9, 10. Stimulation, 2 V, 15 imp/sec.
The vasodilator responses are not abolished, only reduced after atropinization. For explanation, see text.

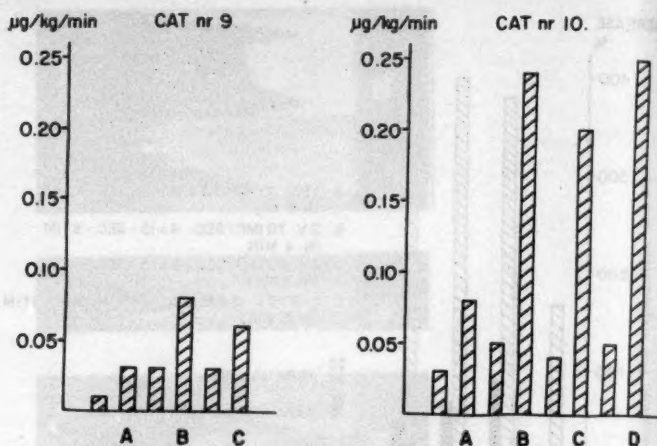


Fig. 7. Absolute values of the adrenaline output in cat no. 9 (Fig. 1) and cat no. 10 (Fig. 5). Note the marked difference in resting level as well as the secretion during stimulation.

is shown in Fig. 7. In the former experiment the adrenaline output increased on stimulation to maximal $0.08 \mu\text{g/kg/min}$, in the latter, to $0.25 \mu\text{g/kg/min}$.

Comments.

Other experiments have shown that intravenous administration of adrenaline in amounts above $0.25 \mu\text{g/kg/min}$ often may elicit vasodilatation in the skeletal muscles (LINDGREN, ROSÉN and UVNÄS 1958). It is not unreasonable to assume, therefore, that the vasodilatation illustrated in Fig. 5 was resistant to atropine because the vasodilator effects were due only in part to activation of the sympathetic vasodilator outflow, and that the occurrence of adrenaline in the blood stream, due to activation of the adrenal medulla, contributed to the vasodilatation. This latter part of the vasodilatation remains, of course, after atropinization of the animal, since the vasodilator action of adrenaline is resistant to atropine.

A further contributory factor in the increased blood flow occurring with stimulation no. 10 in Fig. 5 is the especially marked pressor response initially and terminally. Pressor responses will

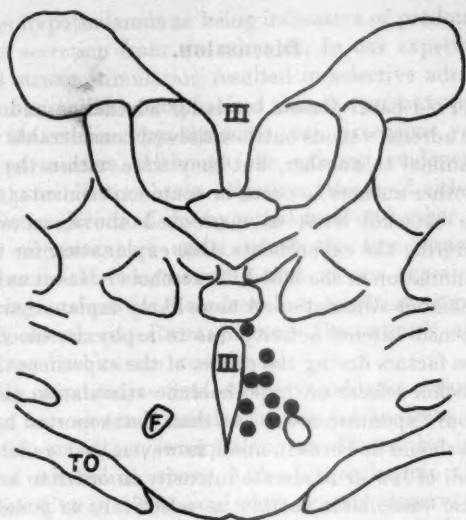


Fig. 8. Drawing of a frontal section through the hypothalamus of a cat's brain, 3-4 mm posterior to the anterior commissure. Stimulation points, histologically localized to a region 1.5 mm anterior or posterior to this section are represented by the black circles.

(F, fornix; TO, optic tract; III, third ventricle.)

cause an increase of blood flow not only for hemodynamic reasons but also by eliciting reflex vasodilatation due to reduction of vasoconstrictor tone. This vasodilatation is of course also resistant to atropine. It should be stressed, however, that such pronounced resistance of the vasodilator response to atropine as in this experiment is exceptional and can usually be avoided by using stimuli of moderate intensity and correctly positioning the electrodes.

Histologic Examination.

The electrode punctures were localized histologically in 12 brains. Nine of the stimulated points were localized in the area corresponding to the frontal chart in Fig. 8. The localization of the responsive points fell within the vasodilator region reported by ELIASSON *et al.* (1951) and LINDGREN *et al.* (1956). Most of the points were localized to the region of the dorsomedial and lateral nuclei in the anterior hypothalamus lateral to the third ventricle and 3-4 mm dorsal to the ventral surface of the brain.

Discussion.

The observed basal plasma levels for adrenaline and noradrenaline in the adrenal venous outflow showed considerable variations from one animal to another, but they were within the limits reported by other authors to occur in acute experiments (see EULER 1956). The catechol level, as mentioned above, increased continuously during the experiments. One explanation for this might be an accumulation in the blood of catechols released as a result of the hypothalamic stimulation. A more likely explanation, however, is a heightened adrenal activity due to asphyxia, blood loss and other stress factors during the course of the experiment.

The catechol release on hypothalamic stimulation that we observed in our experiments was less than that reported by previous authors. It should be borne in mind, however, that we intentionally used stimuli of low or moderate intensity in order to activate the sympathetic vasodilator outflow as selectively as possible.

In the present experiments we consistently observed a marked increase of the adrenaline output (mean 433 per cent). The corresponding increase for noradrenaline was only 84 per cent; in some experiments no increase at all occurred.

FOLKOW and EULER (1954) stated that the adrenaline and the noradrenaline-producing cells of the adrenal medulla had separate innervations and separate representation in the hypothalamus. It should be noted, however, to judge from their published diagram, that most of the points stimulated lay posterior or dorsal to the hypothalamus. Most of the hypothalamic stimulated points yielded a preponderantly adrenalin discharge. A predominantly noradrenaline response is indicated for only one or two true hypothalamic locus. Our results do not contradict such an assumption but suggest that the regions which we stimulated, and which also contain vasodilator neurons, rather selectively innervate the adrenaline-producing cells. A similar selective adrenaline discharge on hypothalamic stimulation was reported by BRÜCKE, KAINDL and MAYER (1952). Since, however, they did not give the exact localization of the stimulating electrodes, a direct comparison of the respective results is not possible.

Our results are in interesting contrast to those of REDGATE and GELLHORN (1953) who interpreted responses of the denervated spleen and nictitating membrane of cats following weak stimula-

tion of the hypothalamus as being indicative of predominant noradrenaline secretion from the adrenals. In our experiments both weak and strong stimulation resulted in selective adrenaline discharge, but it should be emphasized that the present study did not embrace the entire hypothalamus nor extensive variation of stimulus parameters. Although the existing evidence indicates that it is possible to obtain selective release of either catechol amine by stimulation of the hypothalamus and other brain stem areas the circumstances under which the two responses occur remain obscure. It is noteworthy that in our experiments very high resting values for noradrenaline secretion were obtained with Dial anesthesia: REDGATE and GELLHORN's experiments also involved barbiturate anesthesia.

Available quantitative data on the vasodilator action of adrenaline upon the skeletal muscles in the cat indicate that blood vessels with an intact innervation are dilated by intravenous infusion of adrenaline above $0.25-1.0 \mu\text{g/kg/min}$ (LINDGREN, ROSÉN and UVNÄS 1958), and that acutely denervated vessels are dilated by $0.3 \mu\text{g/kg/min}$ (CELANDER 1954). Judging by the present observations, in most of the experiments the amounts of adrenaline which usually attend vasodilator activation by hypothalamic stimulation are too small to cause vasodilatation of innervated muscle vessels. This is already evident from the fact that vasodilatation produced by hypothalamic stimulation usually is completely blocked by atropine. The amounts of adrenaline released are, however, high enough to produce vasodilatation of hyper-responsive chronically denervated vessels (LINDGREN 1955, LINDGREN and UVNÄS, unpublished observations). Only in experiments where the adrenaline discharge is unusually high, or where stimuli of high intensity have been applied, does the adrenaline level reach values high enough to cause vasodilatation in the skeletal muscles.

An adrenaline discharge may influence other vascular areas too, producing *e. g.* vasoconstriction in cutaneous and splanchnic areas. However, sufficiently high adrenaline values have probably been reached only in a few experiments, if any.

In experiments on dogs, WEST and RUSHMER (1957) found that intravenous administration of adrenaline about $0.5 \mu\text{g/kg/min}$ ($0.001 \mu\text{M/kg/min}$) increased the contractile force of the heart. Corresponding data are lacking for the cat, but we find it improbable, judging by the values from dogs, that the amounts of adren-

aline discharged in our experiments can have influenced, except in extreme cases, the contractile force of the heart.

It is difficult to evaluate the biologic significance of our observation that activation of the sympathetic vasodilator outflow is accompanied by a discharge of adrenaline from the adrenal medulla. We have earlier suggested that activation of the sympathetic vasodilator nerves might constitute an integrative part of the autonomic reaction pattern associated with emergency reactions, an opinion also adopted by ABRAHAMS and HILTON (1958).

Worthy of note is that in our experiments we sometimes observed, simultaneously with the vasodilator activation, pronounced outbursts of sham rage with spitting, baring of the teeth, protrusion of the claws, dilatation of the pupils, etc., indicating a reaction pattern characteristic of attack and defense. The vasoconstriction in the cutaneous and splanchnic areas which accompanies the vasodilator responses in the muscles, might result in a redistribution of the peripheral blood flow of the same character as that in muscular exercise. A release of adrenaline in vasoactive amounts should produce similar vascular effects, with vasodilatation in the skeletal muscles and vasoconstriction in the cutaneous and splanchnic areas; it should, in other words, contribute to a suitable readjustment of the peripheral vascular bed.

Even though the amounts of adrenaline released in our experiments were of such low magnitude as to have little if any vascular action, they should have been sufficient to produce metabolic effects aiding emergency reactions.

Circumspection is of course required in drawing conclusions as to the physiologic significance of observations made on anesthetized animals. However, the observation that adrenaline is discharged from the adrenal medulla on hypothalamic activation of the vasodilator nerves to the skeletal muscle lends weight, in our opinion, to our hypothesis that activation of the vasodilator outflow under physiologic conditions is an integral part of emergency reactions. As reported in earlier papers, the sympathetic vasodilator nerves do not appear to take part in vascular reflexes involved in control of blood pressure; these seem to be entirely regulated by variations in the sympathetic vasoconstrictor tone.

Summary.

1. The amount of catechols secreted from the adrenal glands concomitant with activation of the sympathetic vasodilator outflow by hypothalamic stimulation was studied in 14 cats.

2. Blood samples from the left adrenal vein were collected during control and stimulation periods, and were tested (rat uterus and rat colon) after separating adrenaline and noradrenaline by paper chromatography.

3. The mean values for the adrenaline output in the control samples and those taken during hypothalamic stimulation of the sympathetic vasodilator area were 0.06 and 0.32 $\mu\text{g/kg/min}$ (increase 433 per cent). The corresponding figures for noradrenaline were 0.19 and 0.35 $\mu\text{g/kg/min}$ (increase 84 per cent). As will be seen from these values the increase of catechol output consisted predominantly of adrenaline.

4. The conclusion is drawn that in most experiments the amounts of adrenaline released were of such a low magnitude as to have only minor vascular effects, they may have been sufficient to produce metabolic effects.

5. The findings are discussed in view of our hypothesis that activation of the vasodilator outflow under physiologic conditions is an integral part of emergency reactions.

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From the Department of Physiology, Faculty of Medicine (Karolinska Institutet), Stockholm 60, Sweden.

The Presence of the Adrenergic Neurotransmitter in Intraaxonal Structures.

By

U. S. VON EULER.

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Observations of CALABRO (1933), GADDUM and KHAYYAL (1935) and LISSÁK (1939) have indicated that autonomic transmitter substances are present not only in the nerve endings but also in the axones. This was further corroborated by EULER (1951) who found 8.5—18.5 $\mu\text{g/g}$ of noradrenaline in the nerve trunk of the postganglionic sympathetic splenic nerves.

The neurotransmitter is not evenly distributed in the neurone, however, as shown by a comparison of the noradrenaline content of the splenic nerves and the spleen itself. The average noradrenaline content is about 15 μg per g in the nerves freed of their sheath, and about 3 μg per g in the splenic tissue in the cow. These findings indicate that the concentration of the neurotransmitter must be higher in the peripheral parts of the axones than in the nerve trunk, since it is inconceivable that 20 per cent of the spleen should consist of postganglionic nerve axones. No evidence was found for a storage of noradrenaline in the tissue outside the neurones (EULER 1956 a).

Large local differences in noradrenaline concentration within the neurone would hardly be compatible with the assumption that the neurotransmitter is diffusely distributed in the axonal tissue. If the neurotransmitter were manufactured and stored in specific

intraaxonal structures, possibly analogous to the hormone producing and storing microgranules in the chromaffin cells of the adrenal medulla, an uneven distribution pattern could be better explained. An indirect support of this concept is afforded by the close evolutionary relationship between the chromaffin cell and the adrenergic neurone. Moreover, continuous stimulation of adrenergic nerves does not exhaust their stores of the transmitter (ORÍAS 1932), as shown by the persistence of the physiological effects. This suggests that noradrenaline cannot be replaced by the axoplasm flow (WEISS and HISCOE 1948) as this is far too slow to account for a replenishment of released transmitter. It must thus be assumed that noradrenaline is rapidly resynthesized locally in the nerve terminals, possibly in specific structures, also serving as stores. If such hypothetical structures occur, they would be expected to develop in the perinuclear region. This would account for their presence all along the neurone, although there would be no apparent functional need for their presence in other parts than the terminals. An accumulation in the peripheral parts could occur if the storing and synthesizing units are continuously transported towards the periphery by the axoplasm flow.

Assuming a mode of storage of the neurotransmitter of the kind outlined above, it appeared possible to separate the storing units from homogenates of adrenergic nerves. Preliminary experiments have shown that a fraction rich in noradrenaline can be separated from adrenergic nerve homogenates, or from spleen, by high speed centrifugation (EULER and HILLARP 1956). These granules lacked histamine, which occurred abundantly in the supernatant. It was also shown that addition of noradrenaline to a suspension of microgranules from the liver did not result in any transfer of noradrenaline to these granules, indicating the specificity of the catechol containing granules.

The present report describes some further attempts to concentrate the fractions containing the neurotransmitter, and to study some properties of the separated structures.

Material and methods.

Bovine splenic nerves were dissected out and freed from their sheath. The nerves were as a rule collected in ice-cold 0.3–0.88 M sucrose, and in the first part of this study homogenized by grinding in a mortar with quartz sand after cutting them in 3–4 mm pieces.

The homogenate was filtered through gauze cloth and then through filter paper. The slightly opaque suspension was centrifuged in a cold room (0°C) at about $600\text{--}1,000 \times g$ for 5 minutes which brought down a small amount of cell debris and quartz grains. The supernatant was then centrifuged for $1\frac{1}{2}$ —2 hours at $6,000\text{--}10,000 \times g$. The yellowish-pink, homogenous, semitranslucent sediment was as a rule resuspended in sucrose and centrifuged again, and the washing fluid allowed to drain off.

In other experiments the nerves, usually about 5 g, were wrapped in gauze tissue and squeezed between two nylon cylinders. The tissue fluid was collected and the cloth washed with $0.44\text{--}0.88\text{ M}$ cold sucrose and pressed moist dry. For each g of nerve 7.5—15 ml fluid was used. On centrifugation for 5 min at about $1,000 \times g$ the opaque fluid yielded only a minute sediment, containing cell debris, which was discarded. The supernatant was again centrifuged at high speed in the cold and the sediment washed with sucrose. The activity present in the sediment was released by treatment with 1 ml 0.01 N hydrochloric acid per g of nerve and the residue centrifuged off. In some experiments the supernatant before high speed centrifugation was treated with detergents, subjected to freezing and thawing, heating, and to hypotonic solutions as described in the text.

Biological assay.

The biological assay of the solutions was made on the cat's blood pressure, which after pretreatment with 0.1 mg/kg ergotamine tartrate (Gynergen) i. m. and 2 mg/kg atropine sulphate s. c. generally gave a good response to $0.1\text{ }\mu\text{g}$ noradrenaline. In order to test the nature of the sympathomimetic effect on the blood pressure, the solutions were also tested on the isolated chicken rectal caecum, which allows the detection of even small admixtures of adrenaline. The presence of sucrose in the solution did not interfere with the assay, as ascertained by addition of corresponding amounts of sucrose to a standard solution of noradrenaline.

Histamine was estimated on the cat's blood pressure and on the isolated guinea-pig's ileum and further identified by abolition of the effect with the antihistamine Lergigan 1 mg per kg i. m. or by addition to the bath solution to 10^{-4} g/ml . The interference of noradrenaline present in the solution was avoided by treatment of the solution with weak alkali or by addition of manganese peroxide and oxygen, which inactivated the noradrenaline.

Fluorimetric estimation.

When fluorimetric estimation of noradrenaline was made according to the technique described by EULER and FLODING (1956), with the modification that acetate buffer was used at pH 6.5, the figures were considerably lower than those found by biological assay. This seemed

to be due to the suspension medium, since washing the sediment with 0.25 M acetate buffer at pH 6.5 and subsequent treatment with hydrochloric acid at pH 3 or acetic acid at pH 3 gave good agreement between the two methods.

Chromatographic tests.

The activity released from the sediment was subjected to paper chromatography which yielded the typical noradrenaline spot identified by its position, colour reactions and fluorescence in u. v. light.

In order to avoid the sucrose which had a disturbing action on the paper chromatographic procedure, the noradrenaline was adsorbed on alumina and eluted before application to the paper.

Results.

Distribution of noradrenaline in sediment and supernatant of beef splenic nerves.

In a preliminary communication (EULER and HILLARP 1956) it was shown that noradrenaline occurred in the sediment obtained by high speed centrifugation of the cell free suspension from homogenates of rat and cow spleens and cow splenic nerves. After centrifugation for 60 min at $6,800 \times g$ a sediment was obtained which consisted only of fine particles, less than 1μ in diameter, but containing no fibrous structures. After laking the sediment with weak acid the solution showed the typical effects of noradrenaline but displayed no adrenaline action. No other type of biological action could be demonstrated.

Biological assay of the sediment and the supernatant after separation of the sediment confirmed that most of the activity was present in the supernatant. The highest proportion recovered in the sediment in the present experiments was 37 per cent of the total activity of the suspension, but usually it was around 15–20 per cent as in the experiments by EULER and HILLARP (1956). The highest yields in the sediment were obtained by grinding in a mortar with quartz sand, or by squeezing between cylinders, while homogenization in a high speed Waring blender, or according to Potter-Elvehjem, gave a smaller yield. Addition of various factors to the sucrose, such as 0.002 M KCN, iproniazide 0.1 per cent, or sodium ethylene diamine tetraacetate (EDTA) M/150, did not essentially alter the yield in the sediment. The use of Ringer solution instead of sucrose diminished the yield as did preparation at room temperature.

Table I.

Distribution of noradrenaline in sediment and supernatant from homogenates or press juice from cow splenic nerves.

Noradr. in sediment $\mu\text{g/g}$ nerve	Noradr. in supernatant $\mu\text{g/g}$ nerve	Per cent activity in sediment	Noradr. ng/mg dry weight of sediment (normal = 83)	Increase in activity compared with dry weight of nerve	Remarks
1.8	15	11	865	$\times 10.4$	0.3 M sucrose, ground with quartz sand
0.22	1.5	13	320	$\times 3.9$	0.3 M sucrose, homogenized Potter-Elvehjem
5.3	11.2	32	780	$\times 9.4$	0.44 M sucrose, squeezed
3.3	13.5	20	900—1750	$\times 9.8—21$	0.88 M sucrose, squeezed

Activity in sediment.

Table I shows some results as regards distribution of activity, recovery in the sediment and activity of the sediment compared with that of the original nerve tissue, under varying conditions. Using a figure of dry weight for splenic nerves of 18 per cent of wet weight and a noradrenaline content of $15 \mu\text{g}$ per g wet weight, the activity per mg dry weight of nerve is 83 ng/mg. The maximal activity in the sediment was 1,750 ng per mg dry weight of nerve or an increase of about 20 times over the original material.

Distribution of histamine in sediment and supernatant from homogenates of cow splenic nerves.

It has been shown previously that extracts of bovine sympathetic nerves contain large amounts of histamine (EULER 1949, 1956 b), usually around $100 \mu\text{g}$ per g nerve tissue. Most of the histamine present in homogenates of bovine splenic nerves was found in the supernatant after centrifugation. In the sediment obtained by the initial low speed centrifugation and consisting of cell debris, small amounts of histamine were also present, as shown in Table II. However, in the sediment obtained by the following high-speed centrifugation no histamine was found.

The findings suggest that noradrenaline and histamine are bound to different structures in the adrenergic nerve.

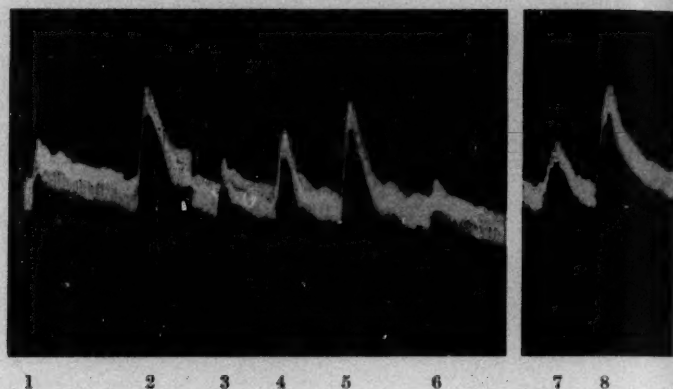


Fig. 1. Blood pressure; cat.

1. 0.1 ml of 30 ml supernatant of press juice in 0.44 M sucrose from 4 g cow splenic nerves after centrifugation 5 min at $1\,000 \times g$.
 2. Same as 1. after addition of 0.01 ml 0.5 N sulphuric acid.
 3. 4. 5. 0.1, 0.15, 0.25 μg noradrenaline.
 6. 0.1 ml 0.44 M sucrose acidified as in 2.
 7. 8. 1/120 and 1/60 of sediment from 1 after 30 min at $10\,000 \times g$ laked in Ringer solution acidified with 0.01 N sulphuric acid (pH 4).
- Ordinate, Blood pressure 120–200 mm Hg.
Time mark, 1 min.

Release of noradrenaline from sediment.

As observed by EULER and HILLARP (1956) the sediment still contains the activity after resuspension in 0.3 M sucrose, showing that the noradrenaline is bound to the sediment.

As stated above the activity in the sediment is released by suspension in 0.01 N hydrochloric acid.

Release of noradrenaline from supernatant after low speed centrifugation (cell-free suspension).

On direct biological assay of the supernatant after 5 min centrifugation at about $1\,000 \times g$ it was observed that addition of acid to pH around 4 increased the activity to a considerable degree. The result seemed to indicate that noradrenaline is partly present in the cell-free suspension in a bound or protected form which can be released by addition of acid.

An example will be given from expt. B4 (Fig. 1). 4 g of splenic nerves were squeezed between nylon cylinders and washed with

Table II.

Noradrenaline and histamine in supernatant and sediments from low and high speed centrifugation of homogenates from cow splenic nerves (μg per g nerve).

Sediment I $1,000 \times g$ 5 min		Sediment II $10,000 \times g^{1/2}$ —2 hours		Supernatant	
Noradr.	Hist.	Noradr.	Hist.	Noradr.	Hist.
< 0.1	3.5	0.22	< 0.1	1.5	65
< 0.02	5.4	0.20	< 0.03	1.8	45
0.07	1.9	0.84	< 0.03	4.0	93
0.1	1.3	1.2	< 0.1	5.2	60

30 ml 0.44 M sucrose and centrifuged for 5 min at about $1,000 \times g$. The small sediment was discarded. The total activity of the supernatant (A) when assayed on the blood pressure of the cat was 30 μg noradrenaline.

After addition of 0.1 ml 0.5 N sulphuric acid per ml to a sample of (A) the activity was increased to 66 μg , indicating that the acid had released a further quantity of 36 μg noradrenaline. Sucrose solution with the same amount of acid had no action.

Supernatant (A) was then centrifuged for 30 minutes at about $18,000 \times g$ and the sediment (1) laked in 6 ml 0.01 N-HCl. The second supernatant (B) contained on direct estimation 34 μg noradrenaline or approximately the same amount as before. On acidifying a small sample of supernatant (B) the activity increased to 50 μg , indicating the release of 16 μg noradrenaline. The sediment (1) contained 12 μg noradrenaline.

The remainder of the supernatant was again centrifuged for 30 min at about $72,000 \times g$ yielding a sediment (2) which contained 7.5 μg noradrenaline. The activity of the supernatant (C) was not increased by acidification, and further centrifugation of the third supernatant at $130,000 \times g$ for 1 hour yielded only 1.7 μg noradrenaline in the small sediment.

From the experiment described above it appears likely that the increased yield of noradrenaline on acidification of the supernatant is largely due to release from the particles which can be sedimented by high speed centrifugation.

Table III.

Effect of various factors on the noradrenaline content in a cell-free microsuspension from press juice of cow splenic nerves in 0.44 M sucrose.

	Un-treated suspension	After freezing and thawing	Heated to 100° C	Acidified pH 3.5 hydrochloric acid	0.001 M cetylpyridinium bromide 1 hour	0.01 M sodium laurylsulphonate 1 hour
Noradr. $\mu\text{g/ml}$	0.33	0.33	0.50	0.70	0.33 0.40 ¹	0.33 0.70 ¹

¹ Frozen and thawed after treatment with the detergents.

While the increase in activity on acidification of the suspension before high speed centrifugation in some cases closely corresponded to the amount found in the sediment, this was not always the case. Thus after squeezing and laking the nerves in Ringer solution in one experiment, the increase on acidification of the supernatant was from 6 to 10 μg per g nerve before high g centrifugation, while the sediment contained only 0.74 μg per g. The supernatant after high speed centrifugation contained 6 μg per g before as well as after acidification. The result suggests that a large part of the particles containing the neurotransmitter has been destroyed and their contents inactivated.

Some factors which have proved efficient in releasing catechol amines from granules obtained from chromaffin cells were tested on the activity of the microsuspension before high speed centrifugation. It was found that while freezing and thawing did not alter the content of the supernatant, a number of other treatments caused a marked increase. This was the case especially after acidification as stated above. Also heating markedly increased the activity. No increase was observed after treatment 1 hour with sodium laurylsulphonate 0.01 M or cetyl pyridinium bromide 0.001 M at room temperature. However, after freezing and thawing a maximal increase occurred in samples previously treated with Na-lauryl sulphonate. A typical example is given in Table III.

Column chromatography.

The extracts obtained by laking active sediments with 0.01 N hydrochloric acid were pooled and adsorbed on aluminium oxide and

eluted with 0.25 N hydrochloric acid. The eluate was concentrated and taken up in 1–2 ml methanol and subjected to chromatography on a starch column using n-butanol-1-N-hydrochloric acid-acetic acid as solvent. The fluorescence of aliquots of the different fractions were determined after condensation with ethylene diamine, and the amounts calculated from the fluorimetric values of standards. As seen in Fig. 2 only noradrenaline could be demonstrated in the chromatogram. The amounts calculated from the fluorescence agreed well with those determined by biological assay of other aliquots of the fractions.

Discussion.

The separation by high *g* centrifugation of a particulate fraction from cell free homogenates of adrenergic nerves, containing as much as 21 times the amount of noradrenaline as in the original nerve material per unit weight, suggests that the neurotransmitter is located to special structures in the axones. These structures have certain features in common with the granules previously demonstrated in the chromaffin cells (HILLARP, LAGERSTEDT and NILSON 1953, BLASCHKO and WELCH 1953). Thus the catechol hormones are not released by laking with neutral 0.3 M sucrose but are liberated by addition of acid to pH 4 or lower. Furthermore, heating, and detergents under certain conditions release the contents.

No other catechols than noradrenaline could be demonstrated in the sediment. This is of some interest since extracts of splenic nerves contain dopamine in similar amounts as noradrenaline (SCHÜMANN 1956, EULER and LISHAJKO 1958), and also other catechols such as dihydroxyphenyl acetic acid (homoprotocatechuic acid). These products therefore seem to be located outside the structures containing the actual neurotransmitter.

On the other hand WEIL-MALHERBE and BONE (1957) reported the presence of large amounts of dopamine in the mitochondrial fraction from 10 per cent sucrose homogenates of iproniazid-treated rabbits' brains. About one half of the adrenaline and noradrenaline in the original homogenate was recovered in the mitochondrial fraction.

SCHMITERLÖW (1948) has presented the hypothesis that the sympathomimetic substance is built up either as such or in the

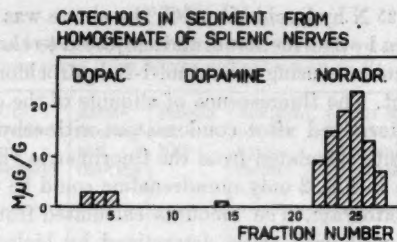


Fig. 2. Extracts of catechols in sediment from cell-free homogenate of cow splenic nerves, subjected to column chromatography in n-butanol-HCl-acetic acid.

form of a precursor in the ganglionic cell (the pericaryon). Since it appeared difficult to explain the accumulation in the nerve endings of the transmitter as such it was believed that it is produced in the form of a precursor which is then transported peripherally in the axoplasm. It was further assumed that a small part of this precursor is changed to noradrenaline already in the postganglionic nerves whereas at the nerve endings this precursor is changed to become the active substance.

The present experiments suggest, however, that special structures are responsible for the synthesis and storage of the transmitter.

In some respects the structures differed from the behaviour of chromaffin cell granules. As reported by HILLARP and NILSON (1954) freezing and thawing released the catechols from the adrenal cell granules whereas this treatment did not liberate noradrenaline from the sediment obtained from nerves or in the cell-free micro-suspension. Laking the sediment in distilled water only released a small proportion of the noradrenaline.

The presence of active substances in intracellular structures has been shown to occur also for histamine and acetylcholine (cf LOEWI 1956, BIRKS and McINTOSH 1957). The presence of the adrenergic neurotransmitter in high concentration in subcellular structures suggests that these serve to store and possibly synthesize the neurohormones. The problem of the physiological release of the neurotransmitter will therefore be intimately connected with the mechanisms by which a release from such structures can be effected in the axon on stimulation.

Summary.

1. In cell-free homogenates of adrenergic nerves in 0.3—0.88 M sucrose, a sediment rich in noradrenaline can be obtained by high speed centrifugation.

2. An active sediment was also obtained by centrifugation of press juice from adrenergic nerves.

3. Noradrenaline was concentrated up to 21 times in the sediment as compared with the original nerve tissue. The highest proportion of noradrenaline recovered in the sediment was 37 per cent of the total activity.

4. While little or no activity was released by suspending the sediment in 0.3 M sucrose, addition of acid to pH 4 or lower released all, and heating most of the activity.

5. Acidification, heating, or addition of detergents followed by freezing and thawing increased the free noradrenaline in the supernatant before, but not after, high speed centrifugation.

6. Histamine was present in the supernatant but not in the sediment containing the noradrenaline.

7. Noradrenaline was demonstrated by column chromatography of extracts of the sediment but no other catechols.

8. It is concluded that noradrenaline is present in high concentration in disperse structures in the adrenergic axones.

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From the Department of Physiology, Karolinska Institutet, Stockholm.

Studies on the Relationship between Olfactory Stimulating Effectiveness and Physico-Chemical Properties of Odorous Compounds.

By

D. OTTOSON.

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All attempts to find a correlation between the physical and chemical properties of odorous compounds and their olfactory stimulative efficiencies have hitherto been unsuccessful. A multitude of theories has been advanced none of which, however, has provided a generally valid explanation. There is a vast literature dealing with this problem and for further introduction the reader is referred to the extensive chapter on olfaction in SKRAMLIK's (1926) textbook and to MONCRIEFF's monograph (1951). The modern literature has recently been reviewed by JONES and JONES (1953).

The lack of knowledge in this field is to a great extent to be traced to the difficulties which are met in attempts to make comparative measurements on the action of different odorous compounds on the olfactory sensory end organs. In studies on the function of other sense organs recording of the impulse discharge in the primary afferent fibres has been a useful means that has provided valuable information. The delicacy of the olfactory nerve fibres (GASSER 1956) has made this approach extremely difficult in the investigations of the electrical activity of the olfactory system

(BEIDLER and TUCKER 1955). The observation that olfactory stimulation evokes slow potential changes which easily can be recorded from the nasal mucosa (OTTOSON 1954, 1956) has, however, made the activity of the olfactory epithelium amenable to direct examination. It therefore appeared to be of interest to study the effect of substances of different characteristics to see whether or not there is a correlation between their stimulating efficiencies and physico-chemical properties.

Methods.

The methods and preparation used in these experiments have been described in detail in an earlier paper (OTTOSON 1956) and will therefore only be briefly dealt with here.

Recordings were made with Agar — AgCl — Ag electrodes from the sensory epithelium in the frog's nasal mucosa. The electrodes were connected to the input of a differential direct coupled amplifier (HAAPANEN 1953). Olfactory stimulation was brought about by blowing a small amount of odorous air on to the nasal mucosa. The duration of the puff of air was about 1 sec and the volume of air blown out 0.5—1 ml. The stimulating substances were generally dissolved in distilled water to obtain suitable stimulus strengths. The purest commercially available compounds were used. The magnitude of the slow potential change evoked in the olfactory epithelium was taken as an index of the stimulatory efficiency of the odorous substance.

Results and Discussion.

The relationship between chemical properties and stimulative effectiveness.

It has long been known that rather few saturated substances are odorous and further that unsaturation leads to an increase of the olfactory effect. This fact has led to the assumption that there is a connection between the ability of a substance to excite the olfactory end organs and the presence of residual valencies in the molecule (WOKER 1906, DURRANS 1919). Thus it has been suggested by DURRANS (1920) that olfactory stimulation is produced by addition reactions. The stimulative effect of saturated compounds was by DURRANS ascribed to the occurrence of a free valency in the oxygen atom in these substances.

In the present investigation the relation between chemical reactivity and olfactory stimulative effectiveness has been studied

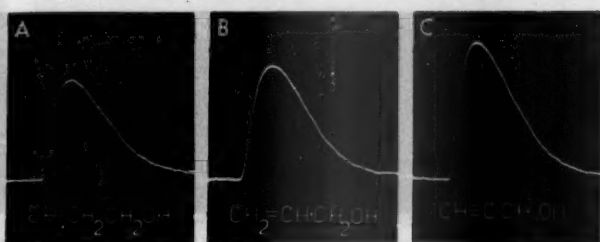


Fig. 1. Olfactory response to propanol (A), allyl alcohol (B) and propargyl alcohol (C).

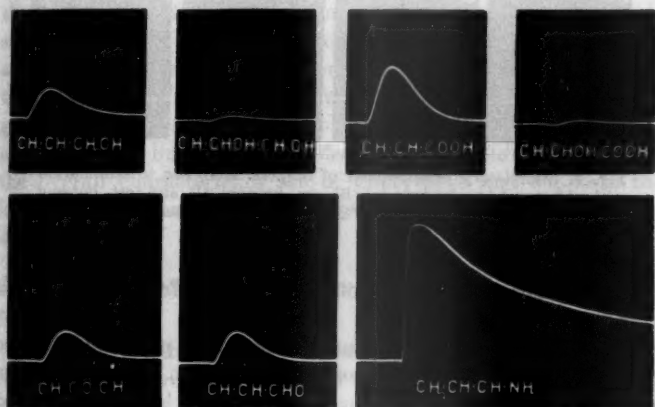


Fig. 2. The effect of introduction of functional radicals. Response to propanol, propylene glycol, propionic acid, lactic acid, acetone, propanol and propylamine.

by using as stimulating agents various unsaturated substances. The effect of gradually decreasing saturation is illustrated in Fig. 1 showing the responses to stimulation with propanol, allyl alcohol and propargyl alcohol. As seen there is an increase in stimulative power with increasing degree of unsaturation. However, the mere fact that a chemically inert substance as propanol elicits a response which is only slightly smaller than that obtained with allyl alcohol rules out chemical reactions as responsible for the excitatory processes. Although the presence of residual valencies enhances the olfactory stimulative effectiveness it is evident



Fig. 3. Response to o-hydroxybenzaldehyde (A) and p-hydroxybenzaldehyde (B).



Fig. 4. Stimulatory effectiveness of isomers. Response to stimulation with n-butanol (A), iso-butanol (B), sec. butanol (C), and tert. butanol (D). Vertical line 2 mV. Time bar 2 sec.

that chemical reactivity is not a prerequisite for a substance to be odorous.

The fact that certain functional groups exert a powerful influence on the olfactory intensity when introduced into the molecule has attracted great interest and a number of theories has been advanced according to which the olfactory effect is attributed to the presence of osmophoric groups (RUZICKA 1920, ZWAARDEMAKER 1922, HENNING 1924, PIRONNE 1929, NICCOLINI 1933). As an example of the effect of various functional groups the records of the responses to some substitution derivatives of propanol are shown in Fig. 2. As seen substitution of H for OH almost abolishes the stimulative efficiency whereas the introduction of the aldehyde or carbonyl group causes a comparatively small change. The greatest effect is produced by the amino group which more than doubles the stimulatory effectiveness. Since the introduction of radicals alters the physical properties of the molecule the change in stimulatory power cannot *a priori* be attributed to the action of these groups *per se*. The position of the radical is of great importance as illustrated by the different stimulative effectiveness of isomers. This differ-

ence is found in aromatic series (Fig. 3) as well as in aliphatic series (Fig. 4). The effect of branching is a decreased stimulatory effectiveness as revealed by the small response evoked by isobutanol (B in Fig. 4) compared with that obtained with butanol (A in Fig. 4). In this connection it has to be mentioned that branched hydrocarbons were found to have less stimulatory effect than straight hydrocarbons on tarsal receptors of insects (DETHIER and CHADWICK 1950). These facts bring up the question whether or not the steric configuration of the molecule is of crucial importance. This matter has been extensively discussed in the literature on olfaction (see *e. g.* MONCRIEFF 1951). Recently it has been taken up again by MULLINS (1955) in an analysis of the factors governing the stimulatory efficiency of odorous substances. From threshold studies in man of odorous compounds of different molecular parameters he inferred that a molecular size corresponding to that of the fourth and fifth member in aliphatic series represents the geometrical optimum. The fact that the olfactory stimulative effectiveness is linked to the molecular architecture suggests that the overall characteristics are of importance and supports the idea that the stimulative processes are of physical nature (WRIGHT *et al.* 1956).

The relationship between physical properties and olfactory stimulative effectiveness.

Homologous substances have generally been used in studies on the relation between the physical properties and olfactory efficiencies of odorous compounds (see *e. g.* BACKMAN 1917, DETHIER and YOST 1952, KRUGER *et al.* 1955). The reason for this is that there is a gradual change in physical properties as homologous series are ascended while the chemical properties remain unaltered. In the present investigation homologous alcohols, aldehydes, ketones and acetates were chosen as test substances. Except for C_6-C_{12} of the alcohols only water soluble members of these series were used. From an earlier study (OTTOSON 1956) it is known that the curve relating the amplitude of the electrical response to the intensity of the stimulus is S-shaped. For the comparison of the stimulative effectiveness of different substances it was therefore necessary to keep the stimulus strengths within the range where the curve has a linear course. Although this was not always possible care was taken to choose for each series of substances a concentration at which the upper limit of this range was not exceeded.

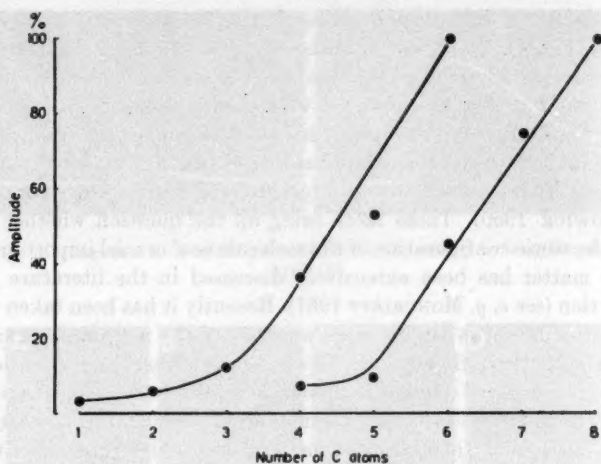


Fig. 5 A. Relation of stimulatory effectiveness of aqueous solutions of primary aliphatic alcohols to chain length. Stimulus strength: 0.01 M for metanol to hexanol (first curve), 0.001 M for butanol to octanol (second curve).

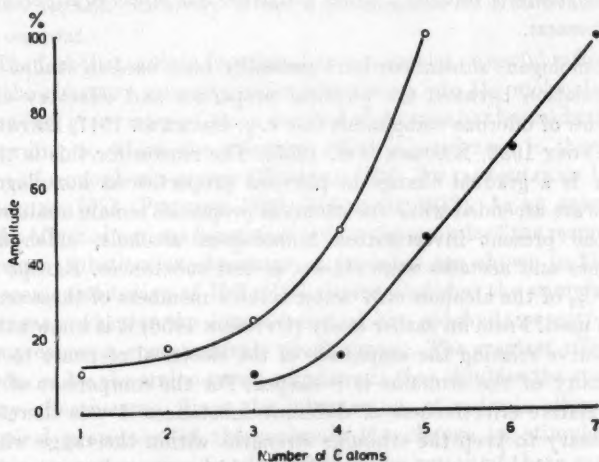


Fig. 5 B. Olfactory stimulative effectiveness of aldehydes (open circles) and ketones (filled circles) in relation to chain length.

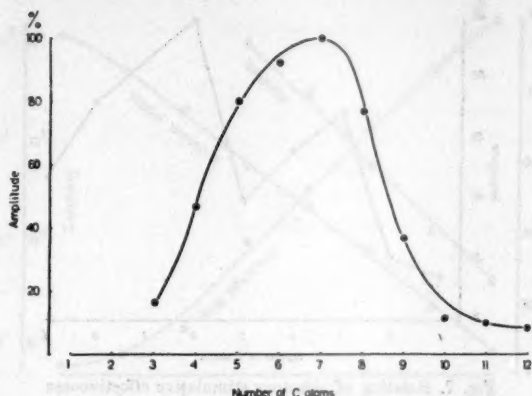


Fig. 6. Relation of olfactory stimulative effectiveness of primary aliphatic alcohols dissolved in oil to chain length.

As shown by the records in Fig. 5 A the olfactory effect of the primary alcohols increased as the series was ascended. When 0.01 M solutions were used stimulation with hexanol gave a response close to the maximal one of the preparation and the higher members in the series had therefore to be tried at a lower concentration (0.001 M). It may be noticed in the first curve in Fig. 5 A that C_5 falls below the line joining the values for the other members. In the search for the reason for this deviation it was found that the stock solution of pentanol used in these experiments was a mixture of n-pentanol and iso-pentanol. From studies on chemoreception in insects (DETHIER and CHADWICK 1950) as well as from the above mentioned observation on the comparative stimulative effectiveness of isomeric alcohols it is known that alcohols with branched chains have less stimulatory effect than those with straight chains. It therefore seems very likely that the comparatively small amplitude of the response to pentanol can be attributed to the admixture of iso-pentanol in the stock solution.

The water solubility of the alcohols decreases as their chains increase in length and they are practically insoluble from C_8 . In order to examine the stimulative effect of the water insoluble members of the series mineral oil was chosen as solvent. The lower alcohols from C_3 which are soluble in water as well as in oil were also included in these experiments. As the series was ascended

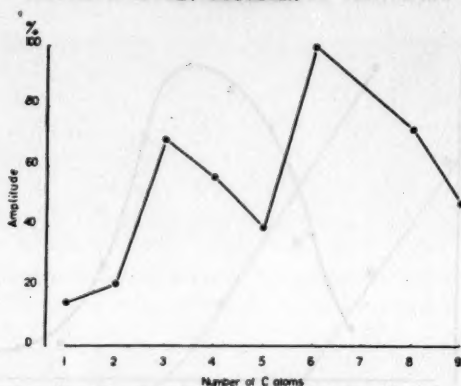


Fig. 7. Relation of olfactory stimulative effectiveness of carboxylic acids to chain length.

the excitatory effect increased but the difference in stimulative power between the members was considerably smaller than that observed when they were dissolved in water. This made it possible to use the same concentration for all the alcohols tested. As shown by the curve in Fig. 6 the stimulatory effect increased gradually from C_3 to C_7 , and then decreased for the higher members. It has to be noted that the greatest response was evoked by heptanol and not by octanol as when water was used as solvent. A corresponding difference, which obviously has to be ascribed to the different action of the solvents, has earlier been observed in studies in human on taste thresholds for alcohols. Thus it was found by DETHIER (1952) that the thresholds for heptanol was lower than that for octanol when the alcohols were dissolved in oil whereas in water solution octanol had a lower threshold.

An increase in stimulative effectiveness with increasing chain length was also found in the other homologous series studied. As shown in Fig. 5 B the olfactory effect of aldehydes (first curve) as well as that of ketones (second curve) increased as the carbon atoms in their chains increased in number. In the series of fatty acids the relation between chain length and stimulative power was less regular (Fig. 7). The underlying reason for this irregularity of the curve is not clear. It may be mentioned, that similar observations have been made by PASSY (1892) and later by BACKMAN (1917) in studies on olfactory thresholds for fatty acids in human.

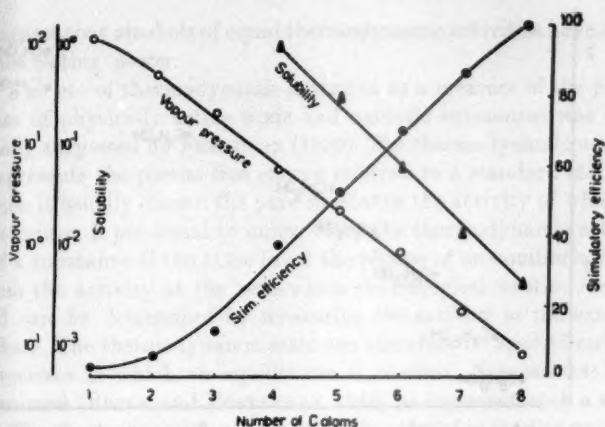


Fig. 8. Relation of olfactory stimulative effectiveness of primary aliphatic alcohols to vapour pressure and water solubility.

BACKMAN ascribed the results to impurities in the test solutions.

As mentioned above, the physical properties of members of homologous series change in an orderly manner as the series is ascended. In Fig. 8 are given the water solubility and vapour pressure curves of C_1 — C_8 of the primary aliphatic alcohols together with the curve for their olfactory stimulative effectiveness. The vapour pressure data are taken from International Critical Tables and from STULL (1947). The solubility values are from Handbook of Physical Chemistry. As shown by the curves the olfactory effect increases as water solubility and vapour pressure decreases. The inverse relationship between water solubility and olfactory stimulative effectiveness was first observed by BACKMAN (1917) who concluded that the action of odorous substances depends on their partition coefficients. Further experimental data supporting the idea that the solubility parameters are of great importance has later been obtained by DETHIER and CHADWICK (1950) in investigations on tarsal reception in bowflies. In the curves relating the stimulative effectiveness to chain length they found a break (CHADWICK and DETHIER 1949) showing a discontinuity in the rate of change. Since this break usually was found in the region where the substances lose their infinite solubility in water it was

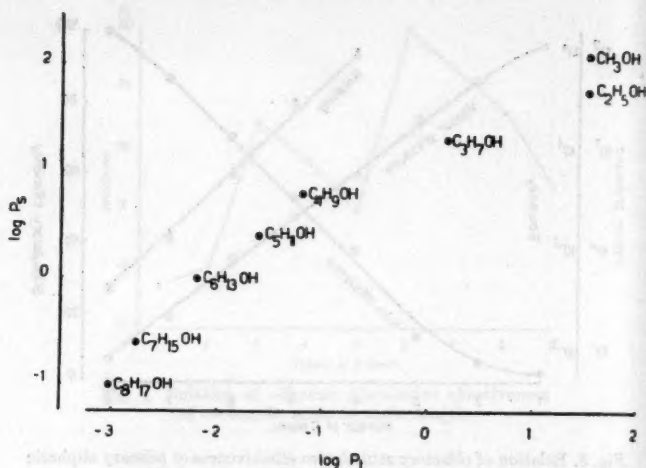


Fig. 9. Relation of partial vapour pressure (p_i) to saturated vapour pressure (p_s) of primary aliphatic alcohols of equal stimulative effectiveness.

concluded (DETHIER 1951) that the limiting mechanism in contact chemoreception involves a two phase system. According to the hypothesis proposed by DETHIER highly water soluble substances stimulate via an aqueous phase and larger molecules via a lipid phase. It is interesting to note that in the curve relating the olfactory stimulatory effectiveness of aliphatic alcohols to their chain length (Fig. 8) there is an inflection in the region corresponding to that where CHADWICK and DETHIER found a break. Regardless of the interpretation of the shape of the curves the similarity between them indicate that the basic mechanisms of excitation in contact chemoreceptors and in olfactory end organs are the same.

In order to evaluate the relation between vapour pressure and stimulative efficiency a series of experiments were undertaken in which by trial and error the concentration of the alcohols were adjusted so that solutions of equal stimulus strengths were obtained. In Fig. 9 the partial pressures of these solutions have been plotted against the saturated vapour pressures. With the exception of the data for the three first members in the series the partial pressures rise almost linearly with saturated vapour pressure. Since the relative vapour pressure (p_i/p_s) for a substance in the vapour phase represents its thermodynamic activity the results

suggest that alcohols of equal thermodynamic activities have equal stimulating power.

The use of thermodynamic activities as a measure of the potencies of physically active toxic and narcotic substances was originally suggested by FERGUSON (1939). The thermodynamic activity represents the partial free energy referred to a standard state. As such is usually chosen the pure substance the activity of which by definition is put equal to unity. Since the thermodynamic activity of a substance is the same in all the phases of an equilibrium system the activity at the locus where the biological reaction is elicited can be determined by measuring the activity in the external phase. The thermodynamic scale can therefore be applied only for processes in which an equilibrium is reached. Narcosis has been assumed (BRINK and POSTERNAK 1948) to represent such a state.

The thermodynamic scale has also been used in studies on olfactory thresholds by GAVAUDAN *et al.* (1948) who found that in terms of thermodynamic activities the stimulating efficiency of aliphatic alcohols increased from C_1 to C_4 and then decreased for the higher members. DETHIER and YOST (1952) used thermodynamic activities as units in investigations on the comparative effectiveness of alcohols in producing repellence in insects. They found all members except the three first in the series nearly equally effective. A similar relation between thermodynamic activities and thresholds was later observed also for the aldehydes and on basis of these observations it was suggested (DETHIER 1954) that homologous compounds of intermediate chain length and of equal thermodynamic activities have equal olfactory stimulative effectiveness.

In view of the linear relationship between the partial vapour pressure and the saturated vapour pressure (Fig. 9) of substances of equal stimulating effect it appeared to be of interest to examine the effect of these substances when used in concentrations corresponding to equal thermodynamic activities. The thermodynamic activities were calculated from the equation $A = f_{\infty} N$ where f_{∞} is the activity coefficient and N is the mole fraction of the substance in solution. The activity value chosen was for all the tested alcohols equal to that of 0.01 M butanol.

The results of these experiments are shown in Fig. 10. The response to butanol has here been taken as 100 per cent. As seen C_1 — C_4 have a considerably lower stimulating effectiveness than butanol whereas the other members produce approximately the same olfactory effect. It is interesting to compare these results

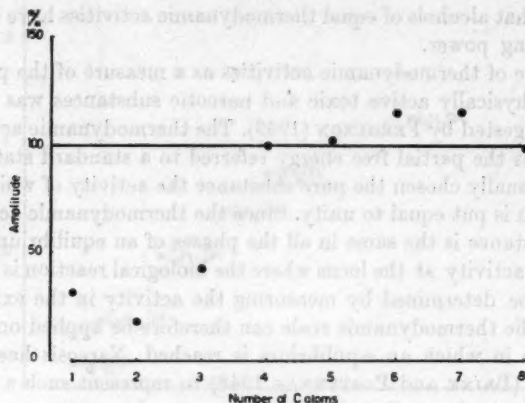


Fig. 10. Stimulative effectiveness of primary aliphatic alcohols of equal thermodynamic activities. Response to butanol taken as 100 %.

with those obtained by DETHIER and his collaborators in their studies in bowflies on the rejection thresholds for alcohols. In bowflies as well as in the frog the three first members in the series of aliphatic alcohols are less efficient than the higher members in stimulating the olfactory end organs. The reason why C_1 — C_3 depart from the C_4 — C_8 is not clear but might be attributed to differences in solubility properties. The lower members in the series are infinitely soluble in water but either insoluble (C_1 and C_2) or only slightly soluble (C_3) in oil. The alcohols in the other group (C_4 — C_8) are on the other hand infinitely soluble in oil but almost insoluble in water. The thermodynamic scale can, as mentioned above, be applied only for processes in which equilibrium is reached. There is at present time no evidence that the processes involved in olfactory stimulation represent such a state. It has been suggested by MULLINS (1954) that the low stimulatory effect of C_1 — C_3 in insects may be ascribed to the fact that equilibrium is not reached between their concentrations in the different phases. The stimulating effectiveness may thus be assumed to be a function of the rate of penetration of the stimulating agent. This view is also supported by the observation that in the series of acetates of equal thermodynamic activities the highly water soluble members have a lower stimulatory effect than the higher members.

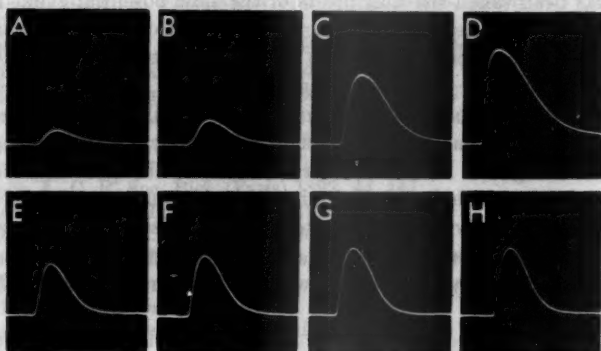


Fig. 11. Olfactory stimulatory effectiveness of acetates (C_1 — C_4). A—D in equimolar solutions; E—H in concentrations of equal thermodynamic activities.

As shown by the records A—D in Fig. 11 the four first members in equimolar solutions give responses which increase in amplitude as the chain increases in length whereas in concentrations of equal thermodynamic activities they have nearly equal stimulating effect (E—H). Methyl-acetate (E) which has a comparatively high water solubility is, however, less stimulating than the other members (F—H).

The experimental data presented above are too scanty to allow any inferences as regards the relationship between olfactory stimulative power and physico-chemical properties of odorous compounds. An analysis of a great number of substances of different characteristics would ideally be expected to give the needed information. However, as long as the basic mechanisms of olfactory excitatory processes are unknown we can hardly hope to understand the differences in action of various odorous agents. Until this gap in our knowledge is filled the relation between the characteristics of odorants and their stimulative effectiveness will remain a challenging problem.

Summary.

1. The relationship between the olfactory stimulating effectiveness and the chemical and physical properties of a number of odorous compounds has been studied. As a measure of the efficien-

cy has been taken the magnitude of the slow potential evoked in the olfactory mucosa in the frog. Aliphatic alcohols of equimolar concentrations were found to be increasingly stimulating up to a certain maximum with increasing chain length. A similar increase was also found for aldehydes and ketones while in the series of fatty acids the relation between stimulative effect and chain length was less regular. It was further found that the partial vapour pressures for alcohols of equal stimulating power increase approximately linearly with the saturated vapour pressures. In terms of thermodynamic activities the alcohols of intermediate chain length are equally stimulating while the lower alcohols are less effective.

2. The relationship between the different molecular parameters and the olfactory stimulative power of the studied compounds is discussed and the similarity between the results obtained in the present investigation and those in studies of olfaction in insects is pointed out.

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From the Department of Pharmacology, Karolinska Institutet,
Stockholm, Sweden.

The Effect of Soybean Phosphatides on the Erythrocyte Sedimentation Rate.

By

BO EDGREN.

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The author has for some time been studying experimentally the possibilities of parenteral nutrition with fat emulsions. In one type of experiment these emulsions were infused intravenously in dogs, and in these experiments it was noticed that the erythrocyte sedimentation rate was substantially lower after the infusion than before.

The aim of the present investigation was to ascertain the effect of intravenously administered emulsions containing neutral fats and soybean phosphatides on the erythrocyte sedimentation rate in the dog. It was found that the soybean phosphatide constituents of these emulsions clearly diminished the sedimentation rate.

Experimental.

The emulsions and solutions employed are shown in Table I.

The soybean phosphatides were purified by the method of WRETLIND (1957) — *i. e.*, dissolving the commercial phosphatides in ether, precipitation with acetone, and drying *in vacuo* — which procedure was repeated three times.

The emulsions were prepared as follows. After heating of all the constituents to 80° C, a coarse emulsion was prepared in a

Table I.

Composition of emulsions and solutions employed in the experiments.

	I. Fat emulsion	II. Phosphatide emulsion	III. Pl F 68 solution
Cotton seed oil ¹	250 g	—	—
Soybean phosphatides ²	12 g	12 g	—
Pluronic F 68 ³	5 g	—	5 g
5 % glucose sol. to	1,000 ml	1,000 ml	1,000 ml

"I Fat emulsion" denotes the 25 per cent cottonseed oil emulsions that were administered in the parenteral nutrition experiments. "II Phosphatide emulsion" contains only soybean phosphatides in an amount equivalent to the phosphatide content of the cottonseed oil emulsions, together with isotonic glucose solution. "III Pl F 68 solution" contains the same amount of polyethylene-polypropylene glycol as that in the fat emulsions, dissolved in isotonic glucose solution.

Waring blender, then homogenized in a Logeman homogenizer for five minutes per 100 ml emulsion. Following this, the emulsions and solutions were autoclaved at 110° C for 12 minutes. — They were stored under nitrogen gas in a refrigerator. Each of the emulsions and solutions employed had been subjected to the same procedure.

The *sedimentation* rate was determined by WESTERGREN's technique. 1.6 ml of blood, taken by venipuncture, was mixed with 0.4 ml of 3.8 per cent sodium citrate solution. The citrated blood was then immediately transferred to a 200 mm Westergren-tube and the red cell sedimentation was read after exactly one hour.

Animal Experiments. In all experiments the emulsions and solutions were administered intravenously in a dose of 14 ml per kg body weight per minute. At this rate the infusions took approximately 1½ hours.

In the first three series of experiments the sedimentation rate was determined immediately before and after the infusions.

1. *Cottonseed Oil Emulsions.* Nine experiments were performed with infusion of cottonseed oil emulsions. Three dogs were used, and they received four, three, and two infusions respectively. The sedimentation rate before and after infusion in each experiment is shown in Fig. 1 : I; the mean value of the results, in Fig. 2 : I.

¹ From Wesson Oil and Snowdrift Sales Co., New Orleans, Louisiana, U.S.A.

² From Glidden Co., Chicago, Ill., U.S.A.

³ From Wyandotte Chemical Corp., Wyandotte, Mich., U.S.A. Pluronic F. 68 is the trade name of polyethylene-polypropylene glycol.

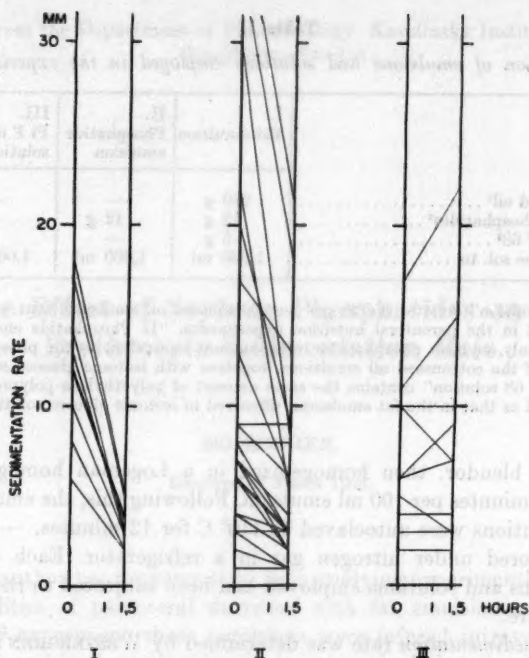


Fig. 1. Influence of fat emulsion, phosphatide emulsion and Pluronic F 68 emulsion on the sedimentation rate in dogs. Ordinate shows the erythrocyte sedimentation rate in mm per hour; abscissa, the time in hours from the start of infusion. The infusion had a duration of $1\frac{1}{2}$ hours, and in each case the sedimentation rate was determined immediately before and after it. Column I relates to the experiments with fat emulsions; II those with phosphatide emulsions, and III those with Pluronic F 68 solution.

2. *Phosphatide Emulsions.* This series totalled 23 experiments on eight dogs, with an approximately even distribution of the infusions. The individual sedimentation rates before and after the infusions are shown in Fig. 1 : II; the mean in Fig. 2 : II.

3. *Pluronic F 68 Solutions.* Ten experiments were conducted in this group, the infusions being distributed more or less evenly among six dogs. The pre- and post-infusion sedimentation rates will be seen from Fig. 1 : III and the mean from Fig. 2 : III.

4. *Complementary Experiments with Phosphatide Emulsions.* In this series the sedimentation rate was studied for a number of hours after intravenous infusion of phosphatide emulsion in five

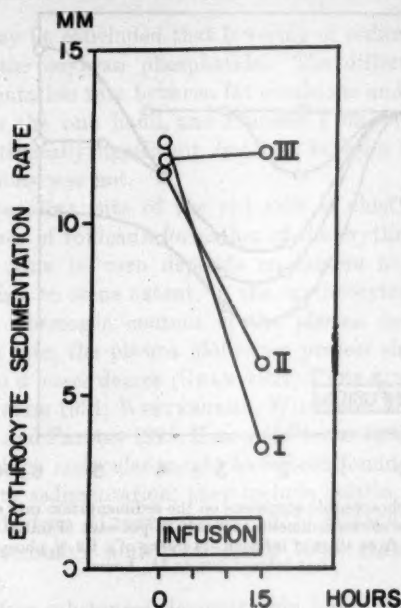


Fig. 2. Influence of fat emulsions, phosphatide emulsions and Pluronic F 68 solutions on the erythrocyte sedimentation rate. Ordinate shows erythrocyte sedimentation rate in mm per hour; abscissa, the time in hours from start of infusion. Curve I shows the effect of infusion of fat emulsions, curve II the effect of phosphatide emulsions and curve III the effect of intravenous infusion of Pluronic F 68 solution. All three curves represent the mean values of the individual results shown in Fig. 1.

dogs. The percentual change was then calculated. The results are presented in Fig. 3.

Results.

It is evident from Figs. 1 and 2 that *fat emulsion* lowered the sedimentation rate. The average fall was from 12.3 mm before to 3.5 mm after the infusions, or a reduction of 72 per cent. A similar reduction was observed after infusion of *phosphatide emulsion*, the sedimentation rate here falling, on the average, from 11.4 mm before to 5.9 mm after the infusions. *Pluronic F 68 solutions*, on the other hand, had little or no effect, the corresponding averages being 11.8 mm before and 12.0 mm after the infusions. In one experiment

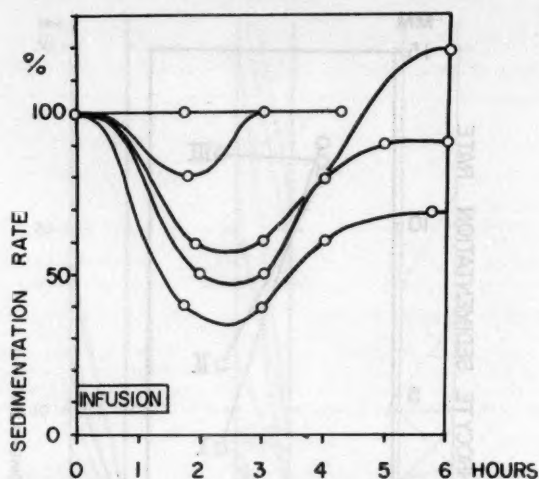


Fig. 3. Effect of phosphatide emulsions on the sedimentation rate during and after infusion. Ordinate shows sedimentation rate in per cent of initial value; abscissa, the time in hours from start of infusion. 14 ml/kg of a 1.2 % phosphatide emulsion was infused during 1½ hours.

the sedimentation rate was so high that the values could not be shown in Fig. 1 (55 mm before and 50 mm after infusion).

In the series in which the sedimentation rate was studied after infusion of phosphatide emulsions, the inhibiting effect of the latter persisted for 2½ hours or more in three of the experiments; in one there was only a slight effect, and in another no effect at all on the sedimentation rate.

Discussion.

It is clear from the results that both fat emulsions containing cottonseed oil, soybean phosphatides and Pluronic F 68 (polyethylene-polypropylene glycol), and emulsions containing only soybean phosphatides, clearly diminished the sedimentation rate in dogs that received the emulsions intravenously in a dose of 14 ml per kg body weight (equivalent to about 0.15 g soybean phosphatides per kg body weight). Solutions of Pluronic F 68 alone in isotonic glucose solution had virtually no effect in this

respect. It may be concluded that lowering of sedimentation rate was due to the soybean phosphatides. The difference in effect on the sedimentation rate between fat emulsions and phosphatide emulsions, on the one hand, and Pluronic F 68 solutions on the other was statistically significant, but that between the two first-named emulsions was not.

The sedimentation rate of the red cells is chiefly dependent upon the degree of rouleaux formation of the erythrocytes (FÄHRÆUS 1921). This in turn depends on factors notably in the plasma but also, to some extent, in the erythrocytes themselves. An increased fibrinogen content of the plasma accelerates the sedimentation rate; the plasma globulines possess similar properties, though to a lesser degree (GRAM 1921, FÄHRÆUS 1929, BENDIEN and SNAPPER 1931, WESTERGREEN, WIDSTRÖM and THEORELL 1931, ZARDAY and FARKAS 1931, HAM and CURTIS 1938). Numerous substances of high molecular weight have been found to accelerate the erythrocyte sedimentation; they include gelatin, pectin, gum arabic, and dextran (LINZENMEYER 1921, HIRSCHBOECK 1947, MLCZOCZ, WUNDERLY and WUHRMANN 1949, HARDWICKE and SQUIRE 1952).

Relatively few substances demonstrably lower the sedimentation rate. The plasma albumin tends to stabilize the red blood cells (COBURN and KAPP 1936) and is considered to have the opposite effect to that of fibrinogen and globulin. KÜRTEN (1920) showed that egg lecithin retarded the sedimentation of erythrocytes *in vitro*. THEORELL (1930) and ZOZAYA (1937) made similar observations, though the former employed synthetic distearyl lecithin.

Soybean phosphatides, purified as described above, contain lecithin, but other phospholipids too (HILDITCH and PEDELTY 1937). Their lecithin content may be the factor that lowers the sedimentation rate; this would be consistent with other observations on the retarding effect of lecithin *in vitro* (KÜRTEN l. c., THEORELL l. c. and ZOZAYA l. c.).

In some of the present cases phosphatide emulsions did not alter the sedimentation rate, although it fell in both earlier and later experiments.

In those experiments with phosphatide emulsions where the sedimentation rates were studied for several hours after the infusions, the retardation generally persisted for some hours. The ultimate normalization of the sedimentation rate was probably

due to elimination of the phosphatides from the blood stream after a few hours.

The cause of the retarding effect of lecithins and soybean phosphatides is obscure. WHITE and MONOGHAN (1936) considered that both the degree of hydration and the electrostatic charge of the erythrocytes had a bearing on the stability of suspension. The erythrocyte stroma contains both phospholipids and proteins. The increase in the stability of suspension may be attributable to a change in the degree of hydration or the electrostatic charge of the red blood cells.

Summary.

Intravenous infusion of fat emulsions, containing cottonseed oil, soybean phosphatides and Pluronic F 68 was found to lower the erythrocyte sedimentation rate in dogs. This effect was shown to be entirely due to the soybean phosphatides.

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